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Binding Domain of the Human Estrogen Receptor by Fluorine  
NMR

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FOREWORD

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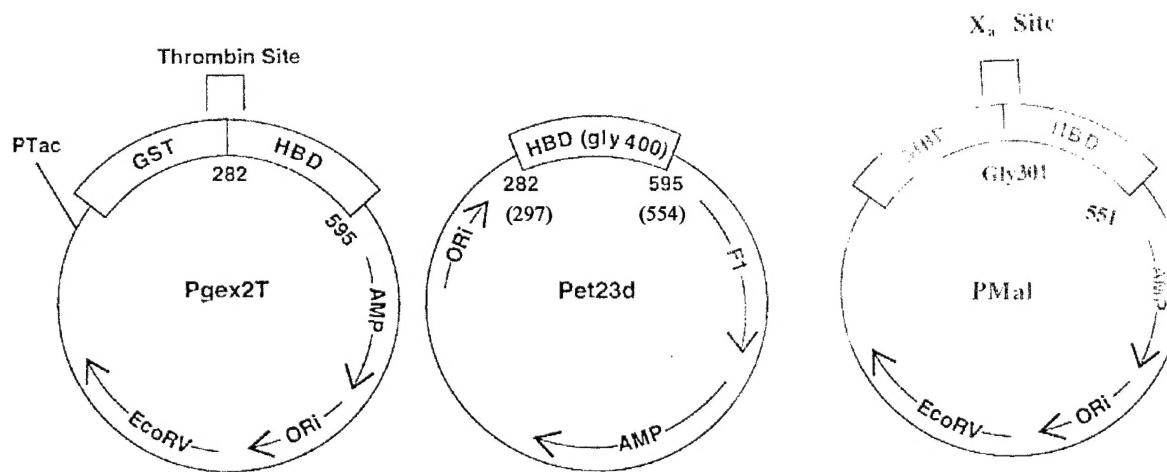
## Introduction

The biological importance of estrogen is noted by its role in development and reproduction and by the number of disease states associated with altered production of estrogen or estrogen like materials. Estrogen is involved in the progression of breast cancer and the estrogen receptor (ER) has been implicated in reproductive cancers. One early step in the process of transcriptional activation by estradiol is conformational changes in the ER upon ligand binding. Our laboratory would like to understand how the structure of the hormone-binding domain (HBD) of the ER changes when it is "activated" by estrogens and antiestrogens. Since the binding of estradiol to its receptor ultimately leads to diverse biological responses to the hormone, we would like to investigate the response of the receptor to miscellaneous ligands and elucidate the molecular basis for their functional differences. This proposal will characterize the critical substrate induced conformational changes in the HBD by incorporating fluorine labels into recombinant constructs and performing fluorine nuclear magnetic resonance (NMR) studies. *The purpose of this proposal is to provide an understanding of the conformational changes in HBD, which will shed light on the molecular events of substrate binding, transcriptional activation and the role of estrogens in receptor function. Our research has shown that conformational changes in the ER are crucial to the function of the receptor and its interaction with associated proteins and nucleic acids.*

## Body

**SYSTEM SET UP.** One of our goals in this study was to produce recombinant HBD with fluorine labels for study by NMR. We used four constructs of the HBD. Figure 1 shows the plasmid maps of three constructs of the hormone-binding domain of the estrogen receptor used in this study. We used two HBD constructs that varied only in the number of residues from Greene's lab as illustrated in figure 1. The first construct, which contains the fusion protein glutathione-S-transferase in tandem with the amino acids 282-595 of the hormone-binding domain of the human estrogen receptor (GST-HBD), was made in the Pgex 2T vector from

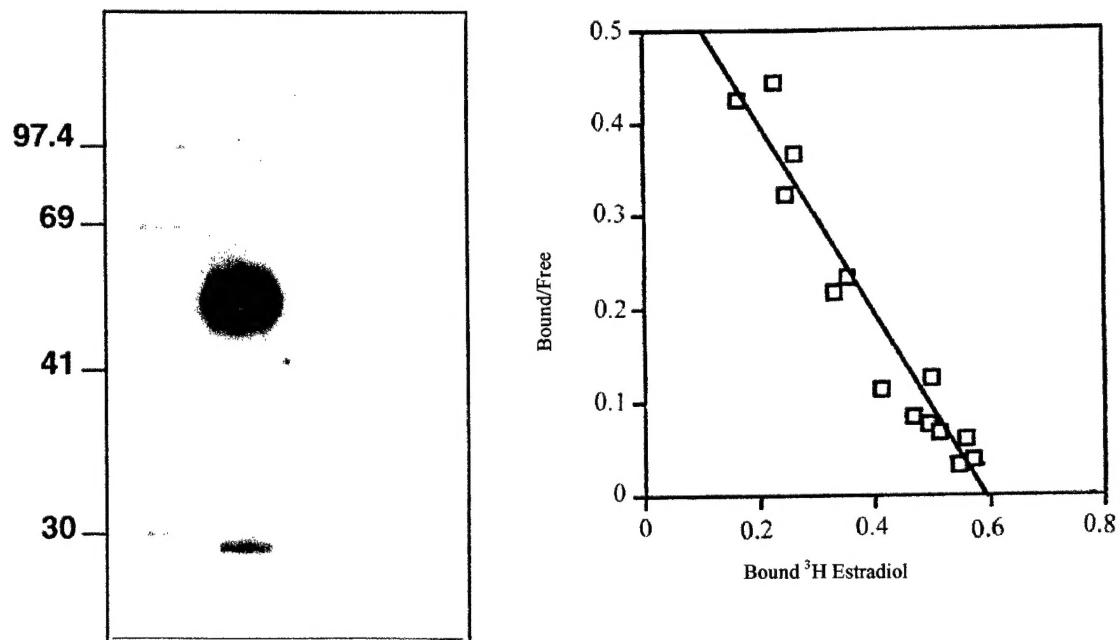
Pharmacia (1). The second construct (GG) was obtained from Geoff Greene's laboratory which contains the residues 282-595 of the HBD in the vector Pet23d from Novagen (2) and the other variation was sHBD, residues 297-554. The third construct was obtained from Mark Brandts and contains the maltose-binding protein in tandem with residues 300-551 of the HBD (MB-HBD) (3).



**Figure 1.** GST-HBD, HBD, sHBD and MBP-HBD expression vectors. The Pgex2T plasmid provides expression of the amino acids 282-595 of the human estrogen receptor in addition to the fused 27kDa glutathione-S-transferase protein, which is used, for protein purification. The Pet23d plasmid generates the HBD (282-595) which we have named GG and HBD (297-554) which we have designated sHBD without additional fused amino acids. This plasmid also contains glycine at position 400. Purification of protein from this plasmid facilitated by an estradiol affinity column. The pMal vector provides expression of amino acids Gly300-551 of the human estrogen receptor in addition to the fused 40kDa maltose binding protein, which is used, for protein purification.

**GST-HBD PROTEIN.** Our first goal was to produce active HBD by means of the GST-HBD vector. To show activity we used the antibody ER 320 from NEO Markers INC (4) and H222 from Greene's laboratory (5) to test for the protein. Both antibodies and ER 311 gave us similar results with our protein preparations. For the most part, we used ER320 antibody since it was available commercially and gave strong signals without background noise. The Figure 2 (left)

shows the Western Blot analysis of the GST-HBD protein. The majority of the protein is shown at ~58 kDa with antibody AER320 which has its epitope in the area of residues 500-595 of the C-domain of the estrogen receptor. The decrease in molecular weight from ~ 62 kDa of the GST-HBD is attributed to proteolysis of the C-terminus of the ligand binding domain that is known to easily cleave to a stable binding core which retains estradiol binding (2b). The thrombin-cleaving site between the fusion protein and the HBD self-cleaves during protein purification to a small degree. Figure 2 (right) shows a  $K_d$  value of 0.9 nM for the typical Scatchard analysis of [ $^3$ H] estradiol binding using low concentrations of (0.1 nM) for the GST-HBD protein.



**Figure 2.** Protein analysis. (A)Western blot analysis of the GST-HBD purification. The H22 antibody was used in an antibody analysis of the purified GST-HBD. Shown in the first lane are the molecular weight markers. A major band is observed at 58kDa and a minor doublet at 30 kDa. (B) Binding of [ $^3$ H]estradiol to labeled GST-HBD. The affinity of unlabeled GST-HBD was determined by the method of Scatchard. (6) Protein concentrations were determined by Bradford method (7) using IgG as the protein standard.

Estradiol binding of the 5F-Trp labeled and unlabeled GST-HBD showed a  $K_d$  of 0.9 nM, which retains the same affinity as the wild type receptor. Since our labeling with 5F-Trp was so low (1-10%) the Scatchard analysis of the GST-HBD protein reflects only the unlabeled protein within the sample.

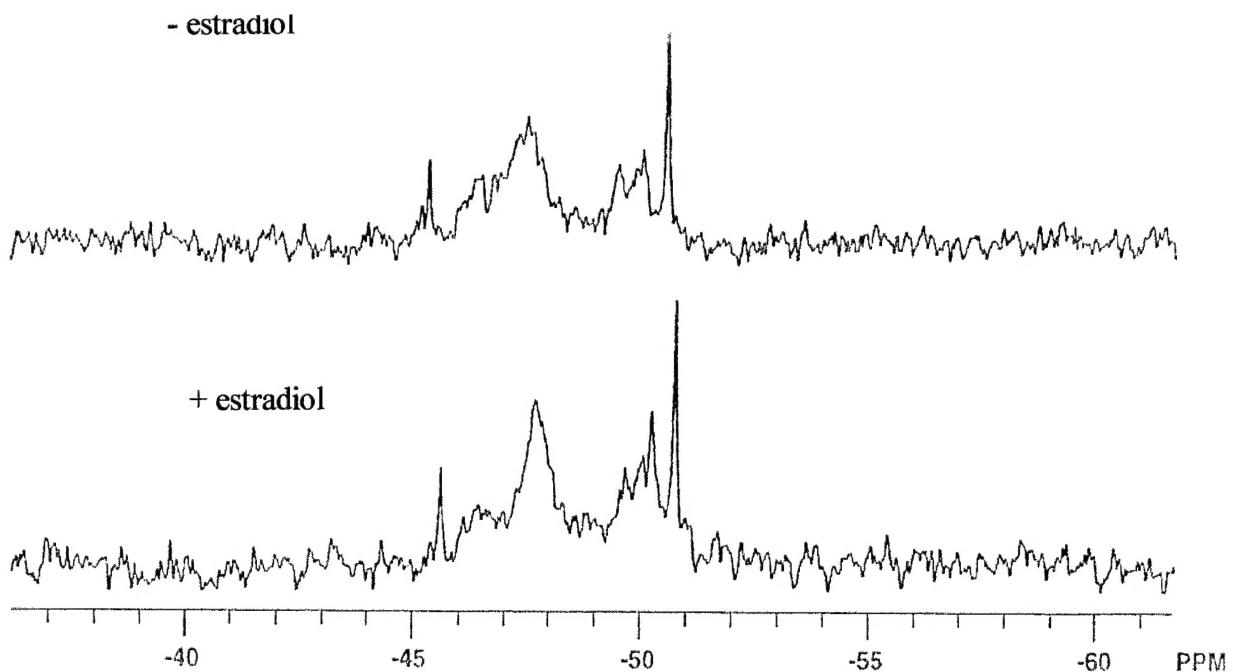
The GST-HBD plasmid was transformed in the W3110 cell line and produced protein. Formulations were investigated to produce protein with 5F-Trp labeling. There are eight Trp residues in the construct, four Trp residues in both the glutathione portions of the protein and the HBD (292, 360, 383, 393). Using a 5:1 ratio of labeled Trp to unlabeled Trp we were able to obtain approximately 5 g of pellet per liter of bacteria. Protein purification was accomplished by means of a glutathione sepharose column and elution with 20 mM glutathione. Extensive dialysis was done after purification.

Fluorine NMR analysis of the GST-HBD showed that the 5F-trp labeling of several preps was efficient to collect data over a 12 hour period. We observed much variation of the labeling efficiency or purification of the protein preparations throughout the time we worked on this particular construct. We also observed that the protein after purification was labile upon freezing and thawing. The spectra below represents many of the preparations of the GST-HBD protein .

Comparison of the protein with and without estradiol shows several differences in the spectra. The top trace in Figure 2 shows the GST-HBD with an empty site for estradiol. This spectrum shows broad lines between -46 ppm and -50.5 ppm. This is consistent with a protein, which is undergoing fluctional changes. The lines are exchanged broadened and indicate that the labeled Trp residues are experiencing numerous conformations within the NMR timescale. There are three Trp residues, which are in positions that are likely to experience conformational change when ligand is added. According to the crystal structure by Siegler (8), Trp 360, 383 and 393 would all be in areas where the ligand would interact with either the residue directly or a helix containing a particular Trp residue. See Figure 4. It is likely that these three residues contribute to the broadened peaks in the spectrum without estradiol. Upon addition of estradiol the peaks at -48.0 ppm becomes more narrowed. The broadened resonances centered about -50 ppm also show a sharped appearance. This sharper appearance is due to the estradiol holding the protein stucture in a more rigid conformation and the Trp residues experiencing fewer conformations.

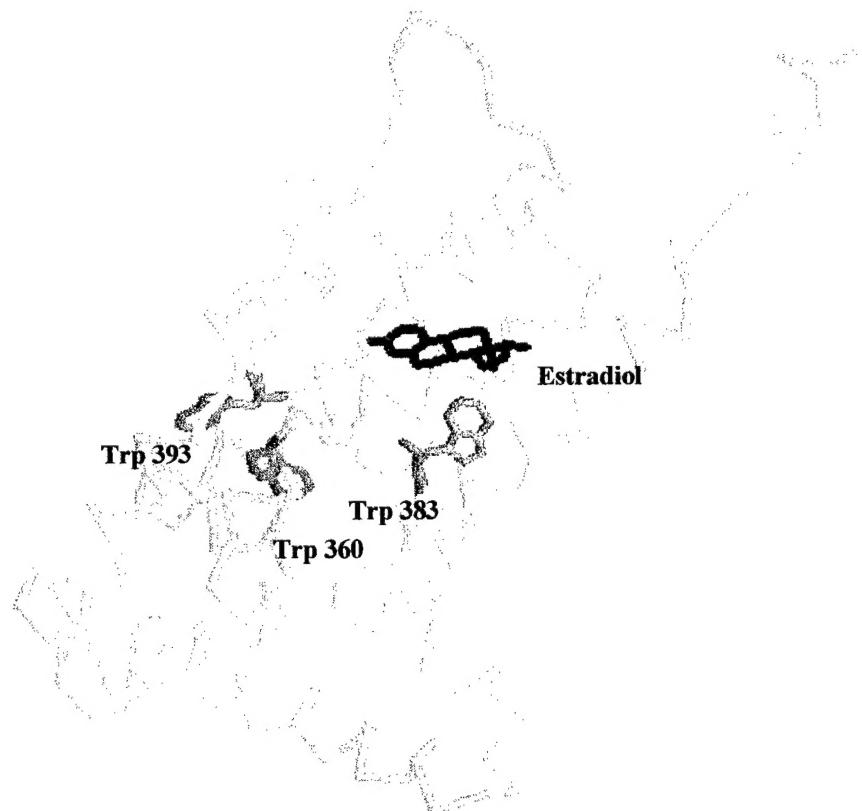
Trp 292, which is not shown in Figure 4, is near the end of the GST-HBD protein construct within 10 residues of the fusion protein. This residue would likely be in a position where it was free to rotate being unconstrained by its normal tertiary and secondary structure in the intact protein. The HBD protein is a truncated version of the ER. The appearance of the narrow peak indicates that the relaxation of the fluorine atom on the Trp residue is slower which may be due to possible solvent exposure. The tentative assignment of this residue to the sharp peak at -45.5 ppm is made due to the peak width. Further evidence for this assignment is justified by the unchanged behavior when estradiol is added to the protein. The spectrum obtained from the labeled protein made from the sHBD construct from Greene's lab further substantiates this assignment. The three residues from this construct show peaks near -50 ppm and there are no residues near -45 ppm. See Figure 10.

The other sharp lines at -51.5 ppm can be assigned to the GST Trp's, two appear in the junction area of the fusion protein make up and one at the opposite end of the protein. The third GST-Trp would be buried beneath the area near -50 ppm.



**Figure 3.** 470 MHz  $^{19}\text{F}$  NMR spectra of 5F-Trp labeled GST-HBD in phosphate buffered saline.

We believe The crystal structure of GST shows all of the four Trp's to be solvent exposed. We originally thought that our sample had PMS (a protease inhibitor added) adducts but mass spectral analysis contradicted this idea.



**Figure 4.** The backbone trace diagram of the HBD residues 297-554 from reference 8. Three Trp residues and estradiol are shown. The arrow points to the helix, which is thought to be out of place due to crystallization procedures.

We can see by these data that there is a conformational change in the HBD protein upon addition of estradiol. The usefulness of the GST-HBD protein as a further investigative probe in this study where we would like to compare ligands is limited. We will not be able to narrow the lines or use site directed mutagenesis to identify specific peaks. The HBD portion of the fusion protein cannot be released from the fusion protein by thrombin due to a secondary cleavage site in the HBD. All attempts to cleave the 5F-Trp labeled protein resulted in non-native protein, which precipitated. Attempts to redissolve the protein from this construct was not successful.

*ASSESSING THE % INCORPORATION OF FLUORINE* We developed a means to determine the % of fluorine incorporation into our proteins using a model protein, the soluble tissue factor (sTF) while working on the HBD project. It served our purposes to find a quick, inexpensive way to get fluorine incorporation data during our protein preparation time rather than wait until the NMR samples were made. The method of choice prior to our method development was to add an internal standard to the NMR sample and determine incorporation by means of integrals and known protein concentrations. This exercise basically gave us information after the fact rather than at a critical time during our protein preparation. In addition this former method, using an internal standard, would not work for the GST-HBD protein since the lines overlapped in the spectrum. Our newly developed method focused on means to monitor small amounts of protein isolated from small-scale bacterial cultures. In this way we could monitor our preps and change the media formulations in response to growth times and % fluorine incorporation.

The development of this new method used a combination of two approaches to assess the level of analogue Trp incorporation into the proteins. The first involved fitting absorbance spectra of the proteins, denatured at neutral pH in 6 M Gdm-Cl by LINCS analysis (8). This approach makes use of the absorption spectra of N-acetyl-Trp-amide, N-acetyl-Tyr-amide and 5F-Trp as basis set spectra for Trp, Tyr, and 5F-Trp residues. To recover the correct Tyr-to-Trp ratio from LINCS analysis of proteins or peptides of known composition containing Trp analogs, such as 5-hydroxytryptophan or 7-azatryptophan, it was necessary to block the  $\alpha$ -amino groups of the analogs. However, 5F-Trp zwitterion in 6 M Gdm-Cl provided a satisfactory absorbance basis spectrum for 5F-Trp-containing sTF.

The second approach to estimation of 5F-Trp incorporation was analysis of the protein mass spectrum by comparing relative peak heights of each appropriate molecular weight species, assuming each 5F-Trp residue will contribute an additional 18 amu to the protein molecular weight. Electrospray mass spectrometry (ESMS) and liquid chromatography ESMS (LC ESMS) were performed and results compared. This work is fully described in reference (9) and another manuscript is in preparation (10).

**Table I.** Estimation of 5F-Trp incorporation in wild type sTF and single Trp-replacement mutants by LINCS analysis and by mass spectrometry.

Protein	LINCS (%)	Mass Spectrum (%)
Wild Type	100	94/100
W14F	77	74
W25Y	72	68/73
W45F	83	73
W158F	79	74

Percent incorporation of 5F-Trp in mutant and wild type proteins was determined as described above. Estimated precision of values obtained from LINCS is  $\pm 8\%$ . Values obtained from mass spectrometry represent results of individual measurements.

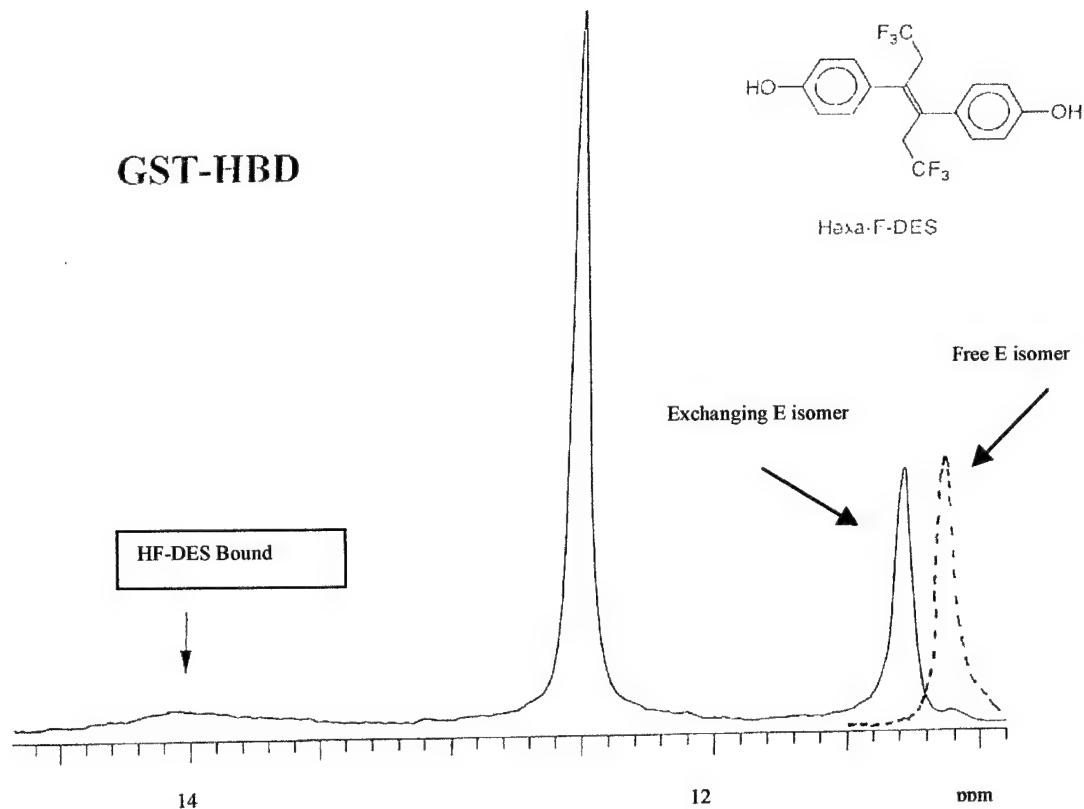
*HEXAFLUORODIETHYLSTILBESTROL ADDITION TO GST-HBD* To further investigate the recombinant protein GST-HBD with environmental estrogens we added hexafluorodiethylstilbestrol (HF-DES) to a unlabeled GST-HBD sample.

Hexafluorodiethylstilbestrol was a generous gift from Louis Levy, NIEHS (11). Figure 5 presents the  $^{19}\text{F}$  NMR spectrum of the protein, GST-HBD and an excess of HF-DES. The resonance for the Z isomer at 12.5 ppm shows no difference in line width or chemical shift frequency from the spectrum of HF-DES without protein (entire spectrum not shown). In

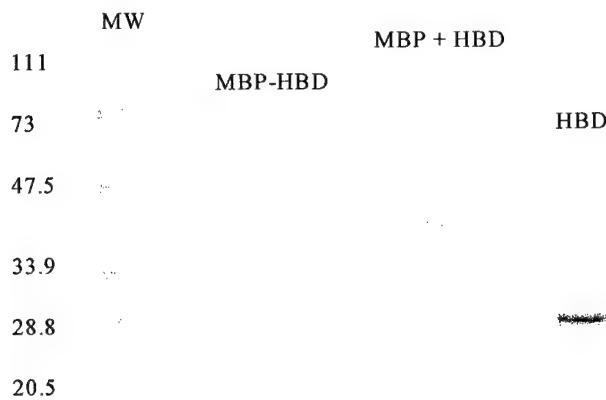
contrast, the E isomer in the spectrum shown at 11.6 ppm is broader by 10 Hz and is shifted by 92 Hz downfield from the free HF-DES in buffer. The dashed lines have drawn the peak for the free HF without the protein in solution. The resonance indicated by the arrow in Figure 5 is present only in the spectrum with bound ligand and protein and represents HF in the binding pocket of the HBD. The intensity and broadness of this peak suggest that the HF-DES is bound in the sterol-binding pocket and is dissociating from the complex at a rate that is the intermediate exchange regime. This spectrum is an example of a protein-induced shift, which arises from the change in environment experienced by the fluorine as it leaves the aqueous phase and enters the receptor-binding site. The most important observation is that the E isomer at 14 ppm is selectively bound in the pocket since the chemical shift of the “free” E isomer which is in exchange with the bound form.

***MBP-HBD CONSTRUCT WITH HF-DES*** Further information concerning protein-induced shifts was obtained using the MBP-HBD construct of the hormone-binding domain of the estrogen receptor. In this experiment we isolated the MBP-HBD protein using the maltose-binding column and then cleaved HBD from the maltose-HBD protein. The SDS page gel of the purification is shown in Figure 6. Our preparation of HBD peptide shows  $K_d$  values of 0.1 nM for estradiol, which is similar to those of the full-length estrogen receptor isolated from mammalian cells. In addition, our peptide has retained structural features required for differential binding to antiestrogens (3).

For the NMR experiment shown in Figure 7 we did not completely isolate the HBD. We cleaved the fusion and protein and left both in solution. The MBP in this experiment was used as a stabilizing agent for HBD. As we have observed in all of our constructs, the HBD alone has a tendency to precipitate. In this experiment the MBP was used as a carrier protein in this experiment. Please note, the HF-DES used in this particular experiment had been stored in the freezer for some time. In storage the Z isomer breaks down because it is less stable and thus our ligand exhibited a 10:1 ratio of E to Z isomer for this particular experiment. The NMR spectrum of the free HF-DES therefore shows a very different appearance from the previous experiment with the GST-HBD protein. We check the ligand for degradation by mass spectrometry. Our experiments concluded that the HF-DES was intact for this experiment.



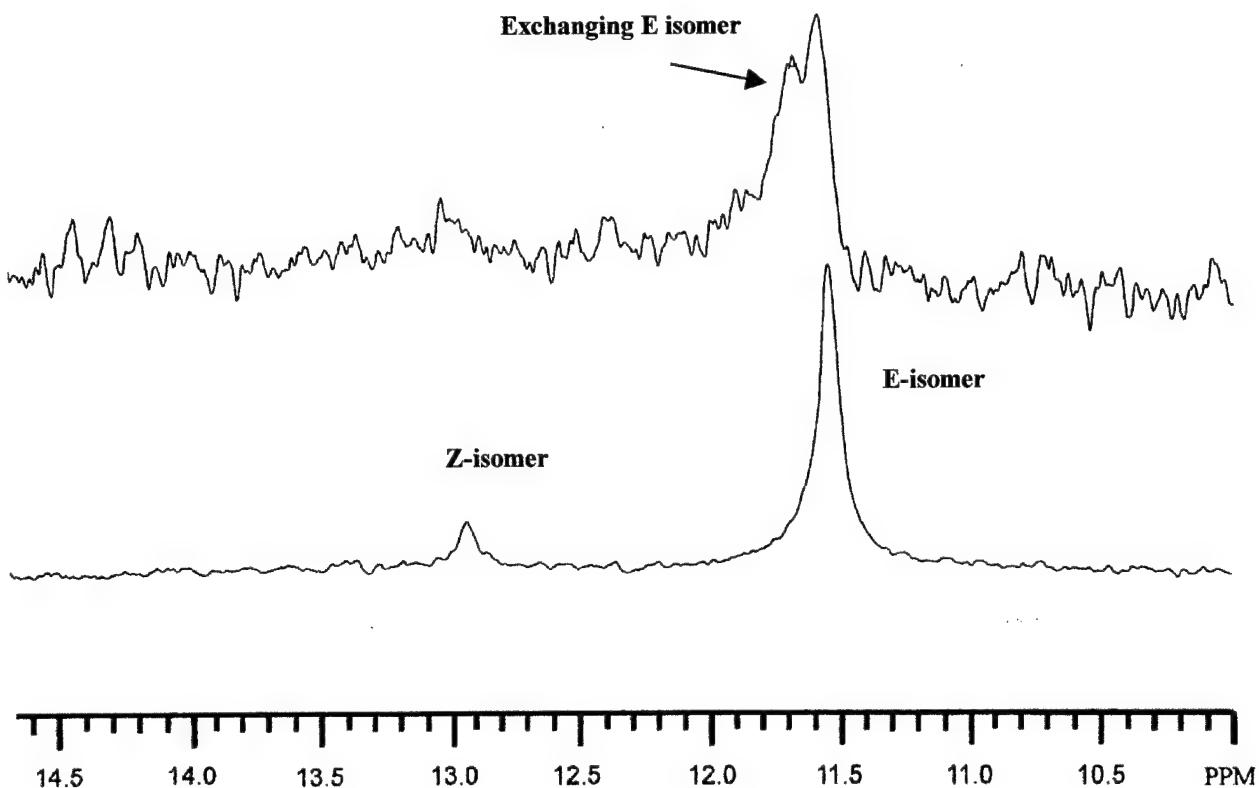
**Figure 5.** 470 mHz  $^{19}\text{F}$  NMR spectrum of 0.25 mM GST-HBD with excess HF-DES in phosphate buffered saline. The arrow points to the bound HF-DES in the sterol-binding pocket. The large peak in the center of the spectrum represents the Z isomer of HF-DES and the dashed line represents the "free" E isomer.



**Figure 6.** The SDS page gel of the purification of HBD from the MBP-HBD protein. The protein for experiment described in Figure 7 is shown in lane 3 (MBP + HBD).

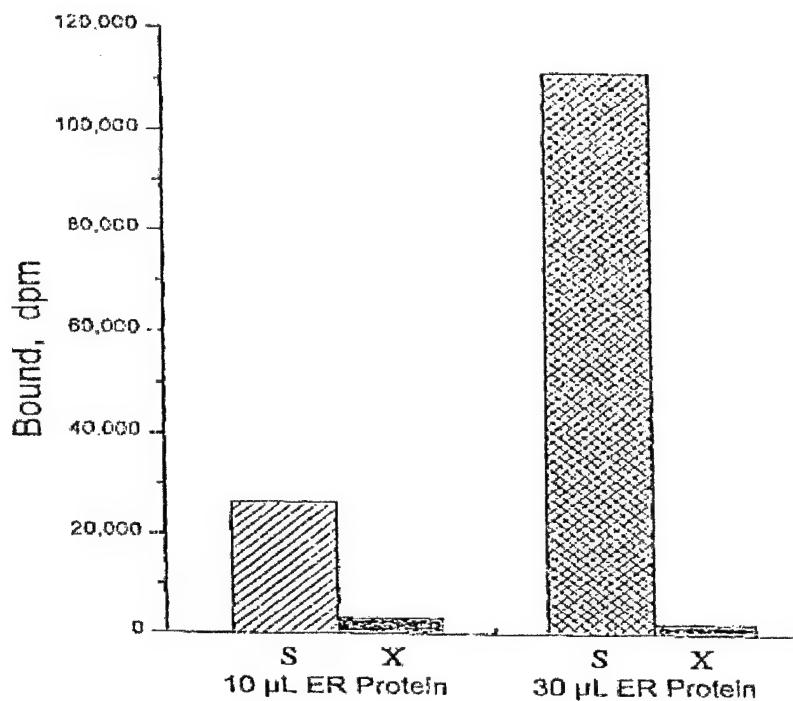
The spectrum of the HF-DES without protein is shown in the bottom trace of Figure 7. When the HBD/MBP protein was added to the HF-DES in a 1:1 ratio we observe a distinct new resonance 0.2 ppm downfield from the E isomer (top trace Figure 7). These resonances at 11.7 and 11.5 ppm indicates the existence of two conditions of the E isomer of HF-DES, one in the free form and the other in equilibrium with a bound form. The two resonances show the same chemical shift differences as the E isomers in the GST-HBD spectrum with HF-DES (Figure 5 dotted and solid lines) We do not observe the low intensity broad peak at 14 ppm in the MBP/HBD experiment. The sensitivity of this experiment was not as good as the previous one with GST-HBD. In conclusion we observe that the *HBD preferentially binds the E isomer*. This work is fully described in reference 12.

Another note: In our previous report (1997) we had mixed up the identities of the Z and E isomers.



**Figure 7.** Top traces the HBD of the human estrogen receptor Gly300-551 with hexafluorodiethylstilbestrol 1:1 mole ratio. Bottom trace hexafluorodiethylstilbestrol without protein.

*HBD and sHBD PROTEIN PRODUCED FROM THE PET23D VECTOR* The protein produced by the HBD construct (282-595) designated GG and sHBD (297-554) is isolated by the use of a estradiol sepharose column provide by the Greene laboratory. The HBD is eluted with estradiol or other substances that will compete with estradiol for the sterol-binding site of HBD. The GG protein shows a doublet around 33kDa on SDS-Page gels and a positive western blot using AE320 or 311 antibodies from Neomarkers Inc. Shown in Figure 8 is <sup>3</sup>H-estradiol binding to HBD and displacement by DES from protein expressed and purified in our laboratory.

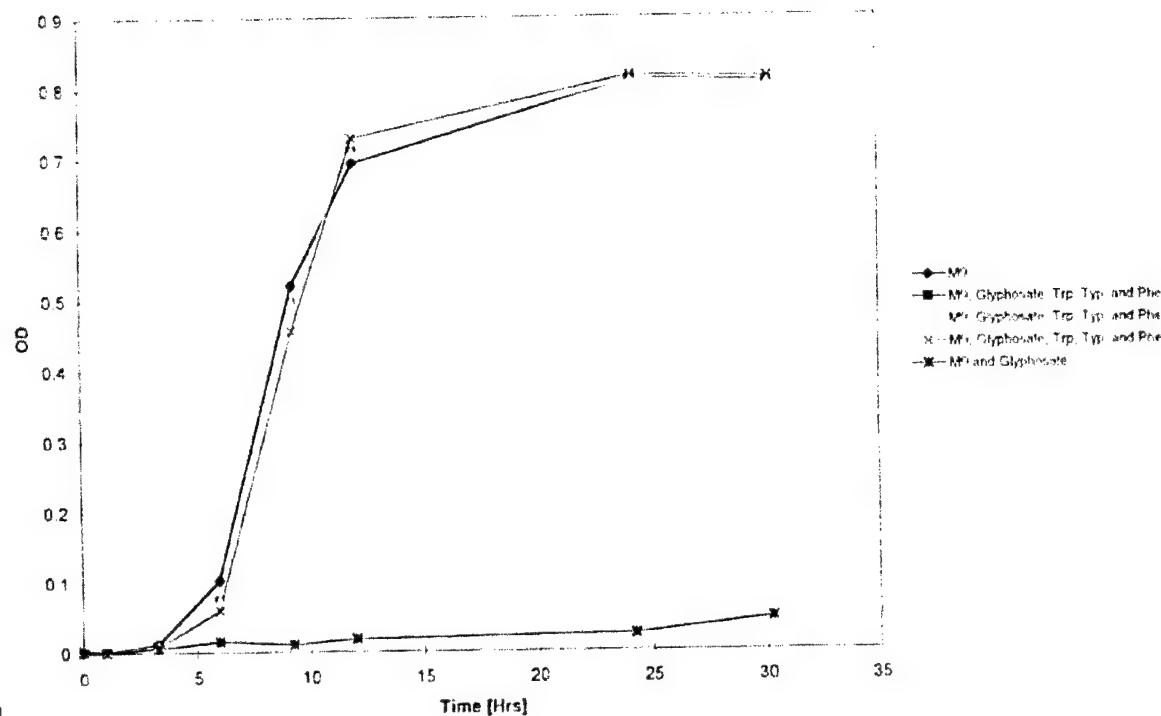


**Figure 8.**  $^3\text{H}$ -Estradiol (3 mM) was incubated in the presence and absence of 200 fold excess of unlabeled DES with 10 or 30  $\mu\text{L}$  of the HBD protein. After incubating for 18 hours at  $4^\circ\text{C}$ , 250  $\mu\text{L}$  of HAP(hydroxyapatite) was added to precipitate the  $^3\text{H}$ -estradiol bound protein. After washing 3 times with buffer, the precipitate was pelleted and counted in a scintillation counter.

Our attempts to transform GG into auxotroph cell lines, W3110, AT2471 and KA197 for Trp, Tyr and Phe respectively did not work. We have found that other constructs using pET vectors were also incompatible for transformation with these cell lines. This was a set back for our efforts and we have been forced to search for alternative methods for obtaining fluorinated proteins from non-auxotroph cell lines. Two such methods are (a) addition of large concentrations of fluorinated amino acids to cultures to flood the cells or (b) adding glyphosate which inhibits aromatic amino acid synthesis and adding the appropriate fluorinated amino acid in addition to the unlabeled amino acids necessary for growth. Monsanto has kindly provided us with gram amounts of glyphosate for our experiments. We have performed growth curves with minimal media, glyphosate and the various labeled and unlabeled aromatic amino acids to quantitate growth and protein production. See Figure 9 for an example of this experiment. Our

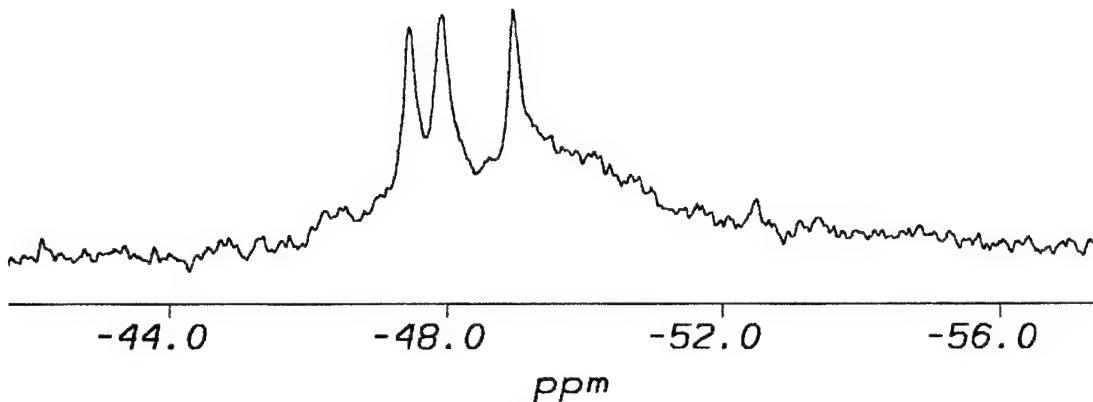
studies have shown that glyphosate with added L-Tyr, L-Phe and 5F-Trp does not inhibit growth of the bacteria and we have been able to get protein production from the plasmid under these conditions. All viable protein from the GG construct that we have isolated has given us mostly unlabeled protein or not fully labeled protein. We originally received a somewhat promising spectrum from the GG construct but felt that our efforts were futile with this construct. Most of the gels showed multiple bands for the protein preparations. Mass spectral analysis and LINCS were also used to analyze these proteins which also lead us to abandon this construct.

We then turned to the sHBD construct. After experimenting with many different preparation we found one that would label the HBD for NMR. The following procedure was used. The bacteria were grown overnight at 37°C in 10 × 1 L of Terrific Broth supplemented additionally with 4 mL/L of glycerol, and the cells were harvested by centrifugation. Each cell pellet from 1 L of culture medium was washed in 100 mL of M9 salts, and then resuspended in 1 L of M9 salts, supplemented with 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 0.04% glucose, 1% casamino acids, 0.1% thiamine and 60 µg/mL ampicillin. The cultures were shaken for 1 hour at 37°C, then transferred to 30°C, supplemented with 60 mg/L 5F-Trp, and shaken for 30 additional minutes. Isopropyl-D-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce protein expression, and the cells were harvested by centrifugation after 4-5 hours of shaking. Most of the HBD prepared was insoluble and would precipitate upon thawing after freezing. We were able to get the following spectrum from over 30 preps of the sHBD (figure 10). This spectrum was run for over 36 hours on a 500 mHz NMR. Our ability to isolate sHBD with other ligands has not been very successful. We also are still working out conditions to remove ligand successfully. When ligand is removed the protein is even more insoluble and we recover even less protein available for NMR.



**Figure 9.** The growth curve of Pet23d in BL21de3 lys cells. The effect of glyphosate on the growth of cells containing the plasmid for HBD.

By the NMR spectrum we can see that there are three resonances corresponding to the 3 Trp residues in the protein. The lines have the same intensity integrating for 1:1:1 areas. Non overlapping peaks indicate that all three residues are in different environments within the protein structure. We confirm this by the X-ray crystal structure seen in Figure 4. The broadened area under the peaks around -50 ppm is expected from unfolded protein. We tentatively assign the peak around -50 ppm to the residue Trp383 that is nearest the binding pocket and the estradiol ligand. Work describing the 5F-Trp labeling of HBD and GST-HBD is in reference 13.



**Figure 10.** 470 mHz NMR of sHBD with estradiol in the binding pocket showing the three available Trp residues (360,383,393). Spectrum was processed with 30Hz line broadening to increase the apparent signal to noise ratio. 4F-Phe was used as an external reference. 15% D<sub>2</sub>O was used as the lock solvent.

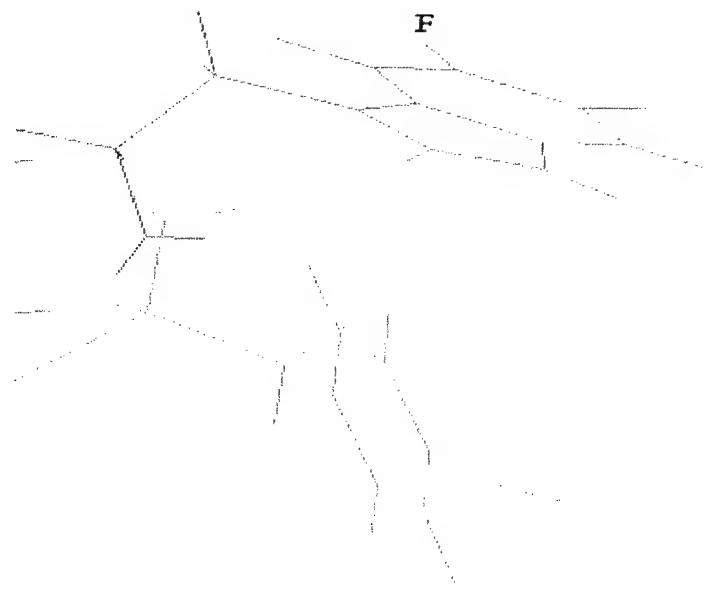
**MBP-HBD PROTEIN WITH LABELED RESIDUES** Our experiments trying to label MBP-HBD have not been very successful. The bacterial strain that produces the proteins does not tolerate the minimal media with the Trp labels. Thus we have had to up the liters of production to get mg quantities of MBP-HBD. Upon cleavage of the fusion proteins we obtain about 75-90% precipitated HBD. The HBD with labels does not resolublize to a great extent. We have however been able to produce <sup>15</sup>N labeled HBD for NMR studies. These are now on going with our collaborators at the Magnet Lab at Florida State University.

**5F-TRP LABELED DBD** Our laboratory has also incorporated 5F-Trp into the DNA binding domain (DBD) of the human estrogen receptor. The DBD was obtained from Ann Nardulli, UIUC (14). We were able to transform the plasmid pT7DBD encoding for residues 180-281 into Trp auxotroph cell line Cy10193. The media used contained M9 plus a mixture of metals

and vitamins with 50 mg of 5F-Trp per liter. Purification was accomplished with a host of protease inhibitors to release the protein from the cells and column chromatography described in reference 11. We were able to assess the fluorine incorporation at 15% by LINCS analysis. The following spectrum was obtained from the 470 mHz NMR experiment see figure 11. In this spectrum there are two peaks indicating that the Trp is undergoing a conformational change. The crystal structure of the DBD (15) shows the one Trp residue in a position that is surface exposed and close to a tyrosine residue. The fluorine on the ring can be close to the Tyr residue or away from the Tyr residue depending on which way the ring is flipped. One position with the fluorine away from the Tyr appears to be more stable (see Figure 12). This stability is reflected in the populations of the two peaks in the fluorine spectrum.



**Figure 11.** The 470 mHz  $^{19}\text{F}$  NMR of 5F-Trp labeled DBD.



**Figure 12.** The Trp-Tyr interaction in DBD. Illustration taken from the crystal structure data in reference 12.

### Key Accomplishments

- Produced Hormone Binding Domain of the Estrogen Receptor with Fluorine Labels
- Demonstrated Conformational Changes in the Hormone Binding Domain with and without Estradiol by Fluorine NMR
- Showed HBD Binds Selectively to the E Isomer of Hexafluorodiethylstilbestrol by Fluorine NMR
- Demonstrated Trp Residues in HBD have Different Environments by NMR

### Reportable Outcomes

#### (A) Manuscripts

Zemsky, J., Rusinova, E., Nermerson, Y., Luck, L.A., Ross, J.B.A. Probing the Local Environments of Tryptophan Residues in Proteins: Comparison of  $^{19}\text{F}$  NMR Results with the Intrinsic Fluorescence of Soluble Tissue Factor Proteins: *Structure, Function and Genetics*, 37:709-716, 1999

Skeels, M.C., Sondi, B.S. and Luck, L.A. <sup>19</sup>F NMR Study of the Binding of Fluorinated Diethylstilbestrol to the Human Estrogen Receptor *Protein and Peptide Letters*, **6**: 149-152, 1999

Luck, L.A., Hulmes, J.D., Rusinova, E., C.L., Crabb, J.W., and J.B.A., Ross. The Use of Mass Spectrometry and Absorption Spectroscopy for Determination of Tryptophan Analogue Incorporation into Proteins to be submitted to *Analytical Biochemistry*

Luck, L.A., Barse, J., Luck, A.M. and Peck C. Conformational Changes in the Human Estrogen Receptor Observed by Fluorine NMR to be submitted to *Protein and Peptide Letters*

(B) Abstracts

Zemsky, J., Rusinova, E., Luck, L.A., Nemerson, Y., Ross, J.B.A. Analysis of Structure and Function of Soluble Tissue Factor Using <sup>19</sup>F NMR and Fluorescence Spectroscopy. Protein Society 11th Symposium, Boston MA, July 1997 Protein Science 6, Suppl. 2: 108, 314-M

Luck, L.A. and Barse, J.L. <sup>19</sup>F NMR Studies of the Recombinant Human Estrogen Receptor Hormone-Binding Domain. Breast Cancer Research Program, Department of Defense U.S. Army Research and Materiel Command. Washington, DC, October 1997

Luck, L.A., Rusinova, E. and Ross, J.B.A. <sup>19</sup>F NMR Studies of 5-F-Tryptophan Labeled Human Tissue Factor. Frontiers of NMR in Molecular Biology VI, Brekenridge, CO January 1999

Luck, L.A., Rusinova, E. and Ross, J.B.A. Solvent Accessibility of TRP Residues in Soluble Tissue Factor: Comparison of Results from <sup>19</sup>F NMR and Fluorescence. Biophysical Society 43rd Annual Meeting. Baltimore, MD February 1999

Luck, LA, Skeels, MC, Sondi, BS, Marin, V, Barse JL <sup>19</sup>F NMR Studies of the Estrogen Receptor 15<sup>th</sup> Regional Cancer Research Symposium. Burlington VT March 1999

Luck, LA, Skeels, MC, Sondi, BS, Marin, V, Johnson, C. and Poteralski, J. JL <sup>19</sup>F NMR Studies of the Estrogen Receptor Northeaster Regional Meeting of the ACS Potsdam, NY June 1999

(C) Invited Seminars

Universitat Potsdam, Germany, November 1996 "Most Recent NMR Studies on Receptor Proteins"

Center for Bioenvironmental Research, Tulane and Xavier Universities, New Orleans, LA, March 1997 "The Biochemistry of Estrogens and Estrogenic Chemicals"

Institute of Molecular Biophysics, Florida State University, 1998 "Fluorine NMR of 5-Fluorotryptophan in Soluble Tissue Factor: Assignment of Resonances by Mutagenesis"

Rowan University, Glassboro, NJ, 1998, "Fluorine NMR Studies of 5-Fluorotryptophan in Soluble Tissue Factor"

Hamilton College, Clinton, NY, 1998, "Fluorine NMR Studies of 5-Fluorotryptophan in Soluble Tissue Factor"

University of Waterloo, Waterloo, Canada 1998, "Comparison of Fluorescence and Fluorine NMR Spectroscopy: A Study of the Soluble Tissue Factor"

Northeast Regional NMR Meeting, 1999, "NMR Studies of Receptor Proteins Labeled with Fluorine"

**(D) Community Service Associated with the Grant**

Keynote speaker, American Association of University Women, St. Lawrence County Branch, 1996, "The Road to Becoming a Research Scientist"

Invited speaker, Breast Cancer Support Group, CVPH Hospital, Plattsburgh, N.Y. 1996, "Research -- Breast Cancer"

Guest of Public Radio, Plattsburgh New York 1996 "Environmental Toxins and Breast Cancer"

Guest Lecture, SUNY Potsdam, 1997, "Environmental Chemicals and Cancer"

Presentation to the Hepburn Group (Clarkson Trustees), 1997, "Research on Breast Cancer"

I worked with Master Gardener Program of Cornell Cooperative Extension over the summer 1999. We displayed posters in the community encouraging homeowner to reduce the use of pesticides and fertilizers.

**(E) Graduate Student Degrees and Projects Associated with this grant**

Vedrana Marin MS 1999 "Incorporation of Trp Labels into Separate Domains of the Estrogen Receptor"

Matthew Skeels "Structural Studies of the Hormone Binding Domain of the Estrogen Receptor" anticipated MS 2000

Branka Sondi "Binding Properties and Solid State NMR Studies of the Leucine Binding Proteins" anticipated MS 2000

(E) Undergraduates and Degrees of Personnel Associated with the Grant

Undergraduate	Degree	Further Degrees/Employment
Colleen Peck	BS Biology 1997	MS Dartmouth 1998 , Research Associate
Jessica Barse	BS Biology/Chem 1997	Proctor and Gamble
Sara Massori	BS Chemistry 1998	UVM Chemistry Ph.D. program
Stephen Brych	BS Chemistry 1998	Florida State Chemistry Ph.D. program
Allison Grenier	BS Biology 1998	UVM Medical School MD program
Brian Knapp	BS Biology 1999	Ohio State Zoology MS program
Jodi Plank	BS Biology 1999	Duke Biochemistry Ph.D. program
Amanda Luck	BS Biology 1999 (Boston College)	
Jesse Poteralski	BS Biology 1999	Wadsworth Institute NY State Health Dept.

(F) Funding applied for based on work supported by this grant

**PENDING AND SUPPORTED**

PRF grant to ACS "Development of New  $^{19}\text{F}$  NMR Probes to Study the Estrogen Receptor with Environmental Chemicals" 1999-2000 \$25,000

NIH "Development of a Piezoelectric Sensor for Estrogens in the Environment" pending \$873,996

**APPLIED FOR AND NOT SUPPORTED**

"Fluorescence and Absorption Studies of Spectrally Enhanced Estrogen Receptors with Environmental Chemicals" US Army 7/96

"Fluorescence and Absorption Studies of Spectrally Enhanced Estrogen Receptors with Environmental Chemicals" Wendy Will Foundation 3/97

"NMR Studies of the Hormone Binding Domain of the Human Estrogen Receptor with Hormone Disrupting Substances" EPA 2/97

"Multinuclear NMR Studies of the Estrogen Receptor" NIH 5/97

" $^{15}\text{N}$ MR Studies of the Hormone Binding Domain of the Estrogen Receptor" Submitted to the US Army IDEA Award with Deborah Wuttke 6/97

Please note that the initial idea and preliminary work on the labeled HBD (produced from the plasmid given to us by Greene's laboratory) was done in my laboratory. It was originally submitted as collaboration then pursued by Deborah Wuttke as sole investigator. Her laboratory

has obtained several grants originating from this idea. Our laboratory is still working on this project with using the MBP-HBD protein.

## Conclusions

Moneys from this grant has supported our work which has resulted in four manuscripts, six abstracts and seven invited scientific talks. There have been a total of 9 undergraduates and 3 graduate students who have worked on this project in my laboratory. Our work has shown the utility of  $^{19}\text{F}$  NMR as a probe for the structure and function of the estrogen receptor. We have illustrated the usefulness of fluorine labeling of the protein and ligand in studies of binding and conformational change in large protein systems. Our studies have also shown that there is a limit to the information that can be gleaned from this protein by studies in solution since the HBD protein is very insoluble. We have shown that the use of a carrier protein will increase the success of the NMR studies but this is also a very limiting factor in full structural studies. Our results can be used in the future as a guide to labeling large proteins and calculating the percent incorporation of the labels. We feel that the estrogen receptor would be a good candidate for solid state  $^{19}\text{F}$  NMR studies. From our work we feel that this would be the most successful route to obtaining information concerning the binding and conformational changes in this protein. These studies could be done on the intact receptor not the domains as separate entities. We have also shown that fluorinated ligands can be useful in obtaining information about binding properties.

## References

- (1) Brown M and Sharp PA. 1990 Human Estrogen Receptor Forms Multiple Protein-DNA Complexes. *J. Biol. Chem.* **265**:11238-43.
- (2a) Seielstad DA, Carlson KE, Katzenellenbogen JA, Kushner PJ, Greene GL 1995 Molecular Characterization by Mass Spectrometry of the Human Estrogen Receptor Ligand-Binding Domain Expressed in *Escherichia coli*. *Mol Endo* **9**:647-658. (2b) Seielstad DA, Carlson KE, Kushner PJ, Greene GL, Katzenellenbogen JA 1995 Analysis of the Structural Core of the Human Estrogen Receptor Ligand Binding Domain by Selective Proteolysis/Mass Spectrometric Analysis *Biochemistry* **34**:12605-12515.

(3) Brandt MA and Vickery LE 1997 Coperativity and Dimerization of Recombinant Human Estrogen Receptor Hormone-binding Domain. *J. Biol. Chem.* **272**:4843-4849.

(4) Abbondaza, C., Falco, A., Nigro, V., Medici, N., Armetta, I., Molinari, A.M., Moncharmont, B., Puca, G.A. 1993 Characterization and Epitope Mapping of a New Panel of Monoclonal Antibodies to the Estrogen Receptor *Steroids*, **58**, 4.

(5) Greene, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., Shine, J. 1986 Sequence and Expression of Human Estrogen Receptor Complementary DNA. *Science*, **231**:1150-4.

(6) Bradford method purchased in kit form from BioRad company.

(7) Scatchard G. 1949 The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51**:660-672.

(8) Tanenbaum DM, Wang Y, Williams SP, Sigler PB. 1998 Crystallographic Comparison of the Estrogen and Progesterone Receptor's Ligand Binding Domains. *1 Proc Natl Acad Sci USA*, **95**(11):5998-6003.

(9) Zemsky, J., Rusinova, E., Nermerson, Y., Luck, L.A., Ross, J.B.A. Probing the Local Environments of Tryptophan Residues in Proteins: Comparison of <sup>19</sup>F NMR Results with the Intrinsic Fluorescence of Soluble Tissue Factor *Proteins: Structure, Function and Genetics* **37**:709-716, 1999

(10) Luck, L.A., Hulmes, J.D., Rusinova, E., C.L., Crabb, J.W., and J.B.A., Ross. The Use of Mass Spectrometry and Absorption Spectroscopy for Determination of Tryptophan Analogue Incorporation into Proteins to be submitted to *Analytical Biochemistry*

(11) Rhee, C.K., Chae, K., Levy, L.A., Korach, K.S. 1995 Synthesis and Estrogen Receptor Binding of Fluorinated Diethylstilbestrol Derivatives. *Bioorg. Med. Chem. Lett.*, **5**, 133-137.

(12) Skeels, M.C., Sondi, B.S. and Luck, L.A. 1999 <sup>19</sup>F NMR Study of the Binding of Fluorinated Diethylstilbestrol to the Human Estrogen Receptor *Protein and Peptide Letters* **6**: 149-152

(13) Luck, L.A., Barse, J., Luck, A.M. and Peck C. Conformational Changes in the Human Estrogen Receptor Observed by Fluorine NMR to be submitted *Protein and Peptide Letters*

(14) Nardulli A.M, Shapiro D.J. Binding of the Estrogen Receptor DNA-Binding Domain to the Estrogen Response Elements Induces DNA Bending. 1992 *Mol Cell Biol* **12**: 2037-42

(15) Schwabe J.W., Chapman, L., Finch, J.T., Rhodes, D. The Crystal Structure of the Estrogen Receptor DNA-binding Domain Bound to DNA: How Receptors Discriminate Between Their Response Elements. 1993 *Cell* **75**:567-578.

24. Kim HW, Perez JA, Ferguson SJ, Campbell ID 1990 The Specific Incorporation of Labelled Aromatic AminoAcids into Proteins Through Growth of Bacteria in the Presence of Glyphosate *FEBS Lett.* **272**:34-36.

## Appendix

### 1. Manuscript

Skeels, M.C., Sondi, B.S. and Luck, L.A. 1999 <sup>19</sup>F NMR Study of the Binding of Fluorinated Diethylstilbestrol to the Human Estrogen Receptor *Protein and Peptide Letters* **6**: 149-152

### 2. Manuscript

Zemsky, J., Rusinova, E., Nermerson, Y., Luck, L.A., Ross, J.B.A. Probing the Local Environments of Tryptophan Residues in Proteins: Comparison of <sup>19</sup>F NMR Results with the Intrinsic Fluorescence of Soluble Tissue Factor *Proteins: Structure, Function and Genetics* **37**:709-716, 1999

### 3. Announcement for Seminar

### 4. Clipping from Plattsburgh Press Republican

## **<sup>19</sup>F NMR STUDY OF THE BINDING OF FLUORINATED DIETHYLTIBESTROL TO THE HUMAN ESTROGEN RECEPTOR**

**Matthew C. Skeels, Branka Salopek Sondi and Linda A. Luck\***

Department of Chemistry and Biology, Clarkson University, Potsdam, NY 13699

**Abstract:** In this paper we report the binding of hexafluorodiethylstibestrol to two constructs of the recombinant hormone-binding domain of the human estrogen receptor produced in *E.coli*. Our fluorine NMR studies have shown the protein to selectively bind the E-isomer and the NMR signals show protein-induced shifts.

### **Introduction**

The actions of estrogenic hormones are mediated through estradiol binding to the estrogen receptor (ER), a member of the superfamily of nuclear receptors [1]. The binding of the estradiol leads to the activation of the receptor, which stimulates transcription at numerous genes containing an estrogen-responsive enhancer element [2]. The biological effects of transcription products are transfixed by the ligand induced conformational changes in the receptor. These structural changes are key to the modulating activities of ER in transactivation, dimerization, chaperone interaction and corepressor inhibition [3]. The ER is known to be promiscuous in its binding property, although agonist and antagonist bind to the same site in the core of the ligand binding domain [4]. A host of hormone mimics that bear no resemblance to estrogen exhibited biological signaling activities in humans and wildlife and have far reaching consequence [5]. One such chemical, diethylstilbestrol (DES), is the archetype of potent synthetic estrogen which was used as a hormone in humans and farm animals [6]. Studies have shown that DES is associated with vaginal cancer, structural abnormalities of the uterus and reproductive dysfunction in female offspring [7]. To understand the molecular and structural details of the interactions between the ER and estradiol, antiestrogens and environmental estrogens we have undertaken innovative NMR experiments utilizing the <sup>19</sup>F nucleus. This paper describes the binding of hexafluorodiethylstilbestrol (HF-DES) to two constructs of the recombinant hormone-binding domain of the human estrogen receptor produced in *E.coli*. According to competitive cytosolic binding studies

the E isomers of diethylstilbestrol have higher affinity for the estrogen receptor than the Z isomers [8]. Upon fluorination of the side chains of diethylstilbestrol there is a change in binding affinities where the fluorinated compounds are slightly lower [9]. This paper addresses the possibility that such fluorinated compounds can be used as NMR active probes for estrogen receptor activity.

### Methods and Materials

The strategy for construction and subsequent protein production from the fusion protein construct, pMal-HBD encompassing the DNA sequence for the maltose-binding protein in tandem with the sequence for residues Gly300-551 of the hormone binding domain of the estrogen receptor has been already described [10]. The DNA construct to produce the fusion protein glutathione-S-transferase with residues 282-595 of the human estrogen receptor was produced by polymerase chain reaction and subcloned in frame into the Bam HI site of pGex-2T (Pharmacia) generating GST-HBD. Sequences were verified using standard dideoxy sequencing of double stranded DNA. Purification of this protein was described previously by Brown [11].

[<sup>3</sup>H]-estradiol binding was performed using aliquots of GST-HBD (0.1pmol) in TEG (40mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 10% glycerol, 1 ug/ul bovine serum albumin which were incubated at room temperature for 60 minutes with varying concentrations of <sup>3</sup>H-estradiol (NEM) with or without unlabeled estradiol. Affinity for estradiol was determined by the method of Scatchard [12]. Western Blot analysis was performed with antibody AER320 on both the GST-HBD and pMal-HBD protein products [13].

<sup>19</sup>F NMR spectra were obtained at 470 MHz on a Varian 500 spectrometer with a 5 mm <sup>1</sup>H/<sup>19</sup>F probe. Samples of GST-HBD in PBS and MAL-HBD in 20 mM Tris-HCl pH 7.8, 1 mM EDTA, 1 mM DTT were prepared with 10% D<sub>2</sub>O as the lock solvent and were referenced to trifluoroacetic acid as an external standard at 0 ppm. Standard undecoupled parameters included 25 Hz line broadening with temperature control at 25°C. HF-DES was obtained from L. Levy at NIEHS and exists in the Z and E forms in our mixture [9].

### Results and Discussion

We have expressed, purified in high yield both the GST-HBD and MAL-HBD proteins. The GST-HBD fusion protein was left intact for our studies but the MAL-HBD was cleaved to HBD peptide according to reference [10]. Both constructs bind HF-DES. The Figure 1 (left) shows the Western Blot analysis of the GST-HBD protein. The majority of the protein is shown at ~58 kDa with antibody AER320 which has its epitope in the area of residues 500-595 of the C-domain of the estrogen receptor. The decrease in molecular weight from ~ 62 kDa of the GST-HBD is attributed to proteolysis of the C-terminus of the ligand binding domain that is known to easily cleave to a stable binding core which retains estradiol binding [14]. Figure 1 (right) shows a K<sub>d</sub>

value of 0.9 nM for the typical Scatchard analysis of [<sup>3</sup>H] estradiol binding using low concentrations of (0.1 nM) for the GST-HBD protein.

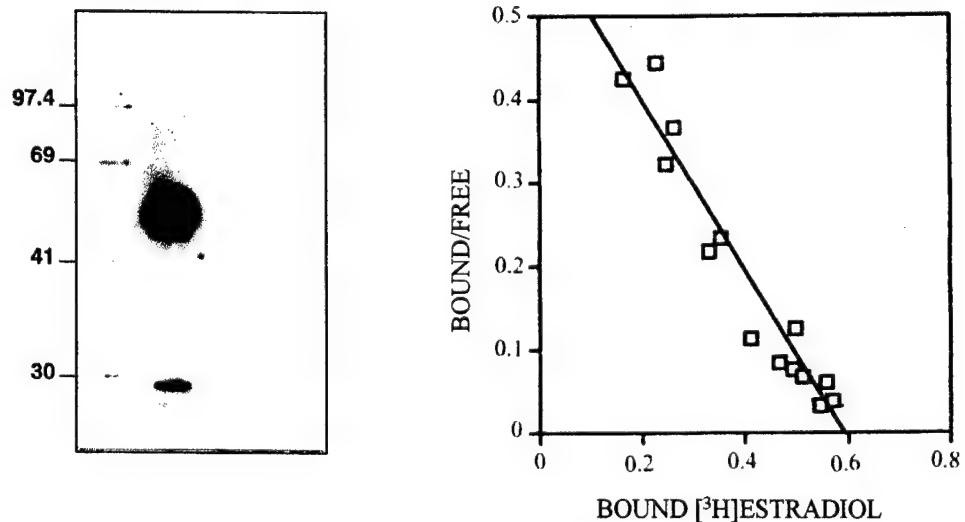


Figure 1. The Western Blot analysis of GST-HBD with antibody AER320 is shown on the left. Molecular weight standards are present in the first lane and the GST-HBD protein in the second lane. Scatchard analysis of GST-HBD is shown on the right.

Figure 2 presents the <sup>19</sup>F NMR spectrum of the protein, GST-HBD and an excess of HF-DES. The resonance for the Z isomer at 12.5 ppm shows no difference in line width or chemical shift frequency from the spectrum of HF-DES without protein (spectrum not shown). In contrast, the E isomer in the spectrum shown at 11.6 ppm is broader by 10 Hz and is shifted by 92 Hz downfield from the free HF-DES in buffer. The peak indicated by the arrow in Figure 2 is present only in the spectrum with bound ligand and protein. The intensity and broadness of this peak suggest that the HF-DES is bound in the sterol-binding pocket and is dissociating from the complex at a rate that is the intermediate exchange regime. This is an example of a

protein-induced shift, which arises from the change in environment experienced by the fluorine as it leaves the aqueous phase and enters the enzyme-binding site. In this spectrum we observe the bound form of the E isomer at 14 ppm and a change in the chemical shift of the "free" E isomer which is in exchange with the bound form. This chemical shift movement could be the result of the alteration in electrostatic interactions, van der Waals interactions, electric fields generated by surrounding dipoles and/or specific hydrogen bonding of the ligand within the binding pocket.

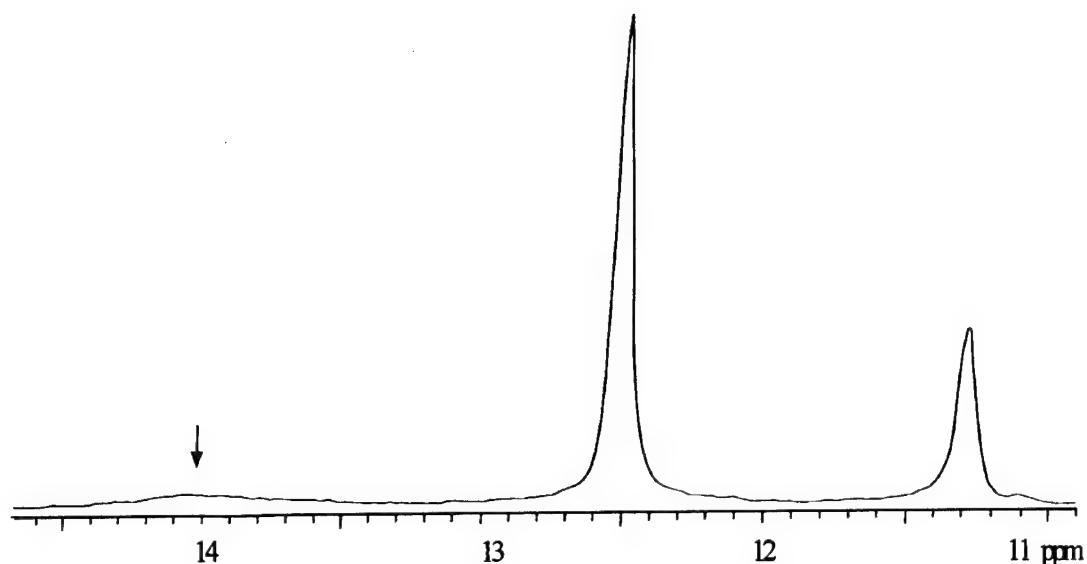


Figure 2.  $^{19}\text{F}$  NMR spectrum of 0.25 mM GST-HBD with 0.5 mM HF-DES in phosphate buffered saline.

Further information concerning protein-induced shifts was obtained using our second construct of the hormone-binding domain of the estrogen receptor. In this experiment we isolated the HBD from the maltose-HBD protein. Our preparation of HBD peptide shows  $K_d$  values of 0.1 nM for estradiol, which is similar to those of the full-length estrogen receptor isolated from mammalian cells. In addition, our peptide has retained structural features required for differential binding to antiestrogens [10]. With the addition of HF-DES that exhibited a 10:1 ratio of E to Z isomer to HBD in a 1:1 equal mixture we observe a distinct new resonance 0.2 ppm downfield from the E isomer (top trace Figure 3). The spectrum of the HF-DES without protein is shown

in the bottom trace. This double resonance at 11.7 and 11.5 ppm indicates the existence of two conditions of the E isomer of HF-DES, one in the free form and the other in equilibrium with a bound form. In this experiment the bound form as observed in Figure 2 at 14 ppm is not resolved. However, the chemical shift of the second E peak is indicative of the E isomer being bound in the ligand pocket.

### Conclusions

We have shown using  $^{19}\text{F}$  NMR and two constructs of the hormone binding domain of the human estrogen receptor that the E isomer of HF-DES binds exclusively. In the future we plan to use these proteins which can be obtained in high yields for further  $^{19}\text{F}$  NMR experimentation. Fluorine-Proton NOE techniques will provide insight into the interactions of this family of ligands and the structural features of this important protein.

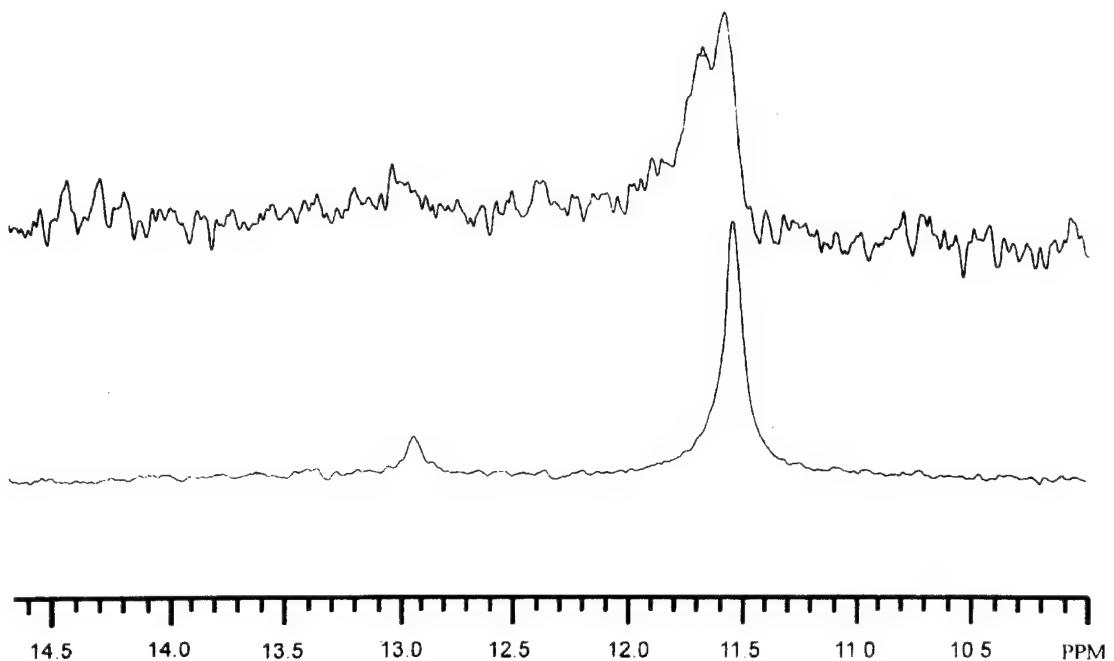


Figure 3. Top trace the HBD of the human estrogen receptor Gly300-551 with hexafluorodiethylstilbestrol 1:1 mole ratio. Bottom trace hexafluorodiethylstilbestrol without protein.

**Acknowledgements**

We would like to thank the US Army Grant DAMD 17-96-1-6140 for financial support of this project. We would also like to thank Louis Levy for his generous gift of hexafluorodioethylstilbestrol and Ken Korach at NIEHS for his helpful discussions. We acknowledge the acquisition of plasmids from both Mark Brandts and Deborah Lannigan.

**References**

- [1] Ciocca, D.R. and Roig, L.M.V. (1995) *Endocr. Rev.*, **16**, 35.
- [2] Tsai, M.J., and O'Malley, B.W. (1994) *Ann. Rev. Biochem.*, **63**, 451.
- [3] Katzenellenbogen, J.A. and Katzenellenbogen, B.S. (1996) *Chem. Biol.*, **3**, 529.
- [4] Brzozowski, A.M., Pike, A.C.W., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J., Carlquist, M. (1997) *Nature*, **389**, 753.
- [5] Cheek, A.O., Vonier, P.M., Oberdorster, B., Burow, C., McLachlan J.A. (1998) *Environ. Health Perspect.*, **106**, Suppl.1, 5-10.
- [6] McLachlan, J.A. ed. (1980) Estrogens in the Environment, Elsevier, New York
- [7] Mori, T. and Nagasawa, H. eds (1988) Toxicity of Hormones in Prenatal Life, CRC Press, Boca Raton, FL
- [8] Korach, K.S., Metzler, M., McLachlan, J.A. (1978) *Proc. Nat. Acad. Sci.*, **78**, 468.
- [9] Rhee, C.K., Chae, K., Levy, L.A., Korach, K.S. (1995) *Bioorg. Med. Chem. Lett.*, **5**, 133.
- [10] Brandt, M.E., Vickery, L.E. (1997) *J. Biol. Chem.*, **272**, 4843.
- [11] Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C., Brown, M. (1994) *Science*, **264**, 1455.
- [12] Scatchard G (1949) *Ann. NY Acad. Sci.* **51**, 660.
- [13] Abbondanza, C., Falco, A., Nigro, V., Medici, N., Armetta, I., Molinari, A.M., Moncharmont, B., Puca, G.A. (1993) *Steroids*, **58**, 4.
- [14] Seielstad, D.A., Carlson, K.E., Kushner, P.J., Greene, G.L., Katzenellenbogen, J.A. (1995) *Biochemistry*, **34**, 12605.

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# CBR

## Center for Bioenvironmental Research Tulane and Xavier Universities

*presents a mini symposium on . . .*

### **“The Biochemistry of Estrogens and Estrogenic Chemicals”**

**Tuesday, March 4, 1997  
9:00 a.m. - 12 Noon**

Room 403, 4th floor

J.Bennett Johnston - Health and Environmental Research Bldg.  
Tulane University Medical Center

#### **Invited Speakers:**

Linda Luck, Ph.D., Associate Professor, Department of Biology and Chemistry,  
Clarkson University

**“Use of fluorine NMR to probe protein structure, ligand binding  
and conformation changes in receptors”**

Manfred Metzler, Ph.D., Professor and Chair, Department of Chemistry,  
University of Karlsruhe

**“The Genotoxicity of Estrogens”**

Harry Jellinck, Ph.D., Emeritus Professor and Chair, Department of Biochemistry,  
Queens University

**“Effect of Indole - 3 carbinole on Estrogen Hydroxylation”**



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# Clarkson professor gets grant to study breast-cancer causes

By JEFF MEYERS  
Staff Writer

PLATTSBURGH — A Plattsburgh native has received a \$150,000 grant to study environmental factors in breast cancer.

Dr. Linda Luck, now an associate professor of biology at Clarkson University, will spend the next two years conducting research on how outside forces may play a role in the formation of cancerous breast cells.

The U.S. Army grant she received is part of a \$100 million project mandated by Congress to address women's health issues.

#### Estrogen reaction

"Basically, we'll be trying to develop ways to screen environmental toxins that affect hormones," said Luck, speaking recently to a local breast-cancer support group. "The main crux of our research will be estrogen receptors, how estrogen reacts with cells."

Estrogen is a substance responsible for the development of secondary sexual characteristics, such as breasts, as females mature. It is a naturally produced hormone but is also made synthetically for therapeutic treatments.

"Estrogen and estrogen-like materials have been shown to be prominent in reproductive cancer," Luck said. "Estrogen is a steroid that diffuses into cells and binds to receptors within the cells."

A receptor is the part of a cell that combines with DNA to trigger a biological response. Synthetic compounds, such as chemicals, can also bind to receptors and create hormonal responses.

When abnormal proteins are created, the cell may become cancerous.



Staff Photo/Jeff Meyers

#### Dr. Linda Luck

"Research is looking at the molecular activity within the cell structure," Luck said. "Cancerous cells are using protein in a way that causes them not to act as normal cells. We want to study how and why that is happening."

#### Cloning

Her new research will look deeply into the cell to study structural changes in estrogen receptors. She'll use a relatively new scientific method where bacteria is used to clone human receptors, a process that makes animal experimentation unnecessary.

The grant will be used to purchase materials, for use of equipment at the University of Vermont and to pay several student research assistants.

#### Eagles and chemicals

Environmental factors in cancer have been studied for more than half a century. In the 1940s, a study on bald eagles in

Florida identified a startling drop in fertility. By the 1950s, scientists believed 80 percent of the state's eagle population was sterile.

Over the next several decades, scientists began making connections between the decline of animal populations and such chemicals as PCBs and DDT.

#### Sperm counts drop

A Danish scientist, Niels Skakkebaek, has conducted a study of human sperm concentrations over the past 65 years. Between 1930 and 1951, concentrations were relatively stable, but in the 1950s levels dropped by 6 percent.

A huge drop of 16 percent was identified in the 1960s, followed by 7- and 5-percent drops over the next two decades.

Experts fear the decline has a direct correlation to our increased use of chemicals and their affects on hormones like estrogen.

#### Reaction

A 1987 breast-cancer study illustrates the threat of hormone-disrupting chemicals. Scientists were studying the effects of estrogen and estrogen-free serum on breast-cancer cells. For some unknown reason, all samples reacted the same despite the level of estrogen added to them.

After months of backtracking, researchers found the culprit: a new plastic resin being used in test tubes was altering the production of protein, augmenting the growth of cancer cells.

#### Pollutants

Luck's new research will examine several kinds of pollutants and how they produce different proteins on the molecular level. The information will add a piece to a puzzle that may some day lead to the prevention of cancers.

# Probing Local Environments of Tryptophan Residues in Proteins: Comparison of $^{19}\text{F}$ Nuclear Magnetic Resonance Results With the Intrinsic Fluorescence of Soluble Human Tissue Factor

Jennifer Zemsky,<sup>1</sup> Elena Rusinova,<sup>1</sup> Yale Nemerson,<sup>1,2</sup> Linda A. Luck,<sup>3\*</sup> and J.B. Alexander Ross<sup>1\*</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, New York

<sup>2</sup>Department of Medicine, Mount Sinai School of Medicine, New York, New York

<sup>3</sup>Department of Chemistry, Clarkson University, Potsdam, New York

**ABSTRACT**  $^{19}\text{F}$  nuclear magnetic resonance ( $^{19}\text{F}$  NMR) of 5-fluorotryptophan (5F-Trp) and tryptophan (Trp) fluorescence both provide information about local environment and solvent exposure of Trp residues. To compare the information provided by these spectroscopies, the four Trp residues in recombinant soluble human tissue factor (sTF) were replaced with 5F-Trp.  $^{19}\text{F}$  NMR assignments for the 5F-Trp residues (14, 25, 45, and 158) were based on comparison of the wild-type protein spectrum with the spectra of three single Trp-to-Phe replacement mutants. Previously we showed from fluorescence and absorption difference spectra of mutant versus wild-type sTF that the side chains of Trp14 and Trp25 are buried, whereas those of Trp45 and Trp158 are partially exposed to bulk solvent (Hasselbacher et al., *Biophys J* 1995;69:20–29).  $^{19}\text{F}$  NMR paramagnetic broadening and solvent-induced isotope-shift experiments show that position 5 of the indole ring of 5F-Trp158 is exposed, whereas that of 5F-Trp45 is essentially inaccessible. Although 5F-Trp incorporation had no discernable effect on the procoagulant cofactor activity of either the wild-type or mutant proteins,  $^{19}\text{F}$  NMR chemical shifts showed that the single-Trp mutations are accompanied by subtle changes in the local environments of 5F-Trp residues residing in the same structural domain. *Proteins* 1999;37:709–716. © 1999 Wiley-Liss, Inc.

**Key words:** 5-fluorotryptophan  $^{19}\text{F}$ NMR; tryptophan fluorescence; tryptophan mutants

## INTRODUCTION

The interactions of proteins with ligands or other macromolecules usually require conformational changes in the protein that also are critical elements in the regulation of biological activity. These structural changes can alter the solvation, electrostatic fields, and van der Waals contacts experienced by particular amino acid residues. As a result, they often can be observed by detecting changes in the spectroscopic properties of specific amino acids. Two methods that have been used extensively to probe protein structure and function are fluorescence, using tryptophan (Trp) residues as intrinsic probes,<sup>1</sup> and  $^{19}\text{F}$  NMR, using

fluorinated amino acids such as 5-fluoro-Trp (5F-Trp) as probes.<sup>2–4</sup> Thus, both fluorescence and  $^{19}\text{F}$  NMR can be used to report on the local environment of Trp residues.

A significant difference between fluorescence and  $^{19}\text{F}$  NMR of proteins is that not all Trp residues necessarily contribute to fluorescence, but all 5F-Trp residues contribute to  $^{19}\text{F}$  NMR. Moreover, changes in local environment are predicted to affect  $^{19}\text{F}$  NMR and fluorescence in subtly different ways. Specifically,  $^{19}\text{F}$  NMR reports perturbations affecting a specific fluorine atom, whereas fluorescence emission reports perturbations affecting the entire indole side chain. The chemical shielding of fluorine is dominated strongly by paramagnetic shifts, and the lone pair electrons make a major contribution to these shifts.<sup>5</sup> The lone pair electrons participate in nonbonded interactions with the local environment. As a result, the  $^{19}\text{F}$  chemical shift is sensitive to changes in hydrogen bonds, electrostatic fields, and van der Waals contacts.<sup>4</sup> In addition, theoretical calculations indicate that an aromatic fluorine is sensitive to changes in the  $\pi$  electron density of the adjacent carbon.<sup>6</sup> Similarly, the delocalized  $\pi$  electron system of the Trp indole ring is sensitive to changes in the local environment, which is reflected in the respective energy shifts (red and blue shifts) of the fluorescence emission of exposed and buried residues. Depending on the position of the fluorine atom on the indole ring and the equilibrium conformations of the entire side chain,  $^{19}\text{F}$  NMR might indicate different solvation or noncovalent interactions for a particular Trp residue than fluorescence does. Combining these two methods should provide a significantly more accurate picture of the local environments of Trp residues of proteins in solution and of the changes in those environments accompanying biologically significant conformational changes than that obtained by either spectroscopy by itself.

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To compare the kinds of information about the local environment of Trp residues obtained from  $^{19}\text{F}$  NMR and fluorescence, we used as a model system soluble human tissue factor (sTF), which has four Trp residues. sTF is a recombinant truncation of tissue factor (TF) that includes the 219-residue extracellular domain of TF, a 263-residue, membrane-bound glycoprotein. After tissue damage, the extracellular domain of TF binds the serine protease factor VII/VIIa, which initiates the extrinsic pathway of the blood coagulation cascade.<sup>7-13</sup> sTF is a particularly useful model for this study because, as we have shown previously,<sup>14</sup> the four Trp residues have distinct absorbance and fluorescence properties, determined from difference spectra of single Trp replacement mutants and the wild-type protein, that can be understood in terms of the local environments determined at high resolution by X-ray crystallography.<sup>15-17</sup> A ribbon diagram indicating the Trp residues in sTF is shown in Figure 1. The absorption and fluorescence emission spectra of multryptophan proteins do not exhibit the resolved contributions of the individual Trp residues even when these residues reside in different local environments. To resolve the individual absorption and fluorescence spectra of each Trp residue, the spectra of single-residue Trp-to-Phe or Trp-to-Tyr replacement mutants were subtracted from the corresponding spectra of the wild-type protein.<sup>14</sup>

Here we report the resonance assignments for each 5F-Trp residue in sTF based on differences between the  $^{19}\text{F}$  NMR spectra of wild-type protein and the same single-Trp replacement mutants. Paramagnetic line broadening and solvent-induced isotope-shift (SIIS) experiments were conducted to assess the solvent accessibility of the 5F-indole side chains. We show that  $^{19}\text{F}$  NMR and fluorescence provide different but valuable complementary information. In particular, the spectrum of a fluorescent residue provides information about the extent to which its indole ring is buried in the protein matrix. By contrast, the  $^{19}\text{F}$  NMR spectrum provides information about the local environment near the position of the fluorine atom on all residues that contain fluorine. Thus, from  $^{19}\text{F}$  NMR it is possible to determine for a Trp residue that has fluorescence reflecting partial exposure of the indole ring, which part of the indole ring is either shielded by the protein matrix or exposed to solvent. We show also that 5F-Trp  $^{19}\text{F}$  NMR spectra can reveal subtle perturbations in the local environment of the NMR probe associated with single Trp mutations within the same protein domain. These mutations, however, neither result in discernable changes in protein activity nor perturb the fluorescence properties of the individual Trp residues.

## MATERIALS AND METHODS

### Reagents

5-DL-Fluorotryptophan was from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI) and Acros (Pittsburgh, PA). Recombinant human VIIa was a generous gift from Novo Nordisk (Denmark), and factor X was purified from human plasma by published methods.<sup>18,19</sup> 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylserine and 1,2-dioleoyl-*sn*-glycero-3-pho-

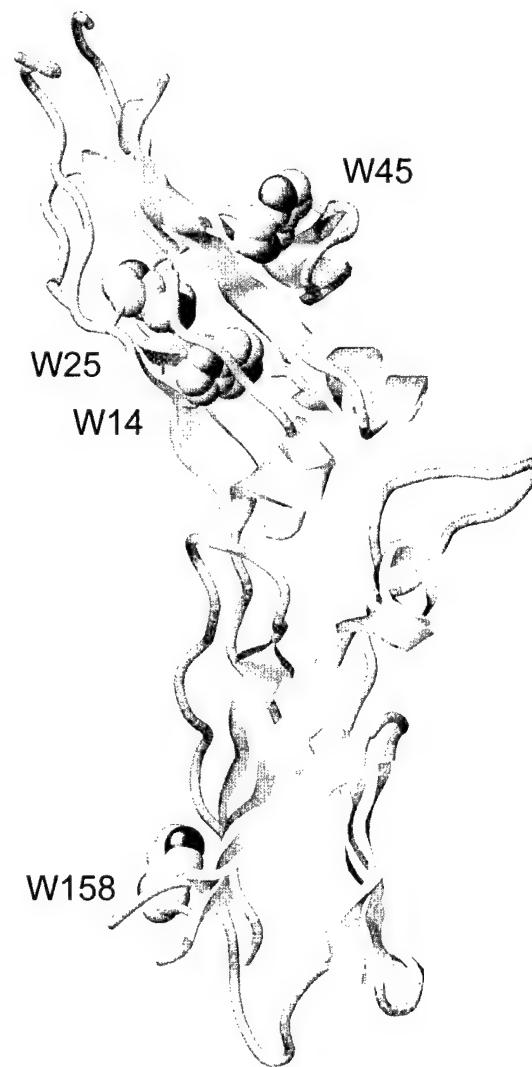


Fig. 1. Rasmol<sup>31</sup> view of sTF based on the 1.7 Å resolution X-ray crystal structure by Muller et al.<sup>16</sup> The gray ribbon indicates the main chain of sTF, and the four Trp side chains are shown as space-filling representations. The fluorinated atom on each indole ring is C-5, which is black.

photidylcholine were from Avanti Polar Lipids (Alabaster, AL). The chromogenic substrate Spectrozyme was from American Diagnostica Inc. (Greenwich, CT). Guanidinium chloride (Gdm·Cl) was from Heico Chemicals (Delaware Water Gap, PA). Deuterium oxide ( $\text{D}_2\text{O}$ ), gadolinium (Gd), and diethylenetriaminepentaacetic acid:gadolinium (III) dihydrogen salt dihydrate (Gd:DTPA) complex, were from Aldrich (Milwaukee, WI).

### Expression and Purification of Wild-Type and Mutant Proteins

Wild-type and mutant plasmids (W14F, W25Y, W45F, W158F) were the same as previously described.<sup>14</sup> Following standard protocols,<sup>20</sup> the plasmids were transformed into the *Escherichia coli* tryptophan auxotroph CY15077ΔEA2.<sup>21</sup> The resultant strains were grown overnight at 37°C in 6 × 1 L of Terrific Broth supplemented

with an additional 4 mL/L of glycerol, and the cells were harvested by centrifugation. Each cell pellet from 1 L of culture medium was washed in 100 mL of M9 salts and then resuspended in 1 L of M9 salts supplemented with 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 0.04% glucose, 1% casamino acids, 0.1% thiamine, and 60 µg/mL ampicillin. The cultures were shaken for 1 h at 37°C and then transferred to 30°C, supplemented with 50 mg/L 5F-Trp, and shaken for 30 additional min. Isopropyl-D-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce protein expression, and the cells were harvested by centrifugation after 4–5 h of shaking.

The complementary deoxyribonucleic acids (cDNAs) for sTF, three Trp-to-Phe mutants (W14F, W45F, and W158F), and one Trp-to-Tyr mutant (W25Y) were constructed with a leader sequence that directs the protein to the periplasmic space of *E. coli*. Previously, the proteins were purified from concentrated media after induction at 20°C overnight.<sup>14</sup> With the shorter induction period used here, most of the expressed protein is retained in the cells. Therefore, the proteins were released from the periplasmic space by osmotic shock by using the following protocol. The cell pellets were resuspended in 1 L of 30 mM Tris, pH 8, 20% sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), and stirred for 5 min at room temperature. The cells were pelleted by centrifugation, and the supernatant was discarded. The pellet was resuspended in 1 L of ice-cold 5 mM MgSO<sub>4</sub> and stirred for 5 min at 4°C (adapted from Snavely et al.<sup>22</sup>). The lysed cells were pelleted and discarded, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the supernatant to 65% saturation. The remaining purification steps were essentially as described previously.<sup>23</sup>

The yields of proteins expressed in the *E. coli* Trp auxotroph CY15077ΔEA2<sup>21,24</sup> varied in a mutant-dependent manner. For example, the yields from 6 L of bacterial culture were about 65 mg for wild-type sTF but only about 1 mg for mutant W14F and much less for mutant W25Y. Unfortunately, the quantity of W25Y recovered was insufficient for NMR. It should be noted that the same levels of protein expression were observed, whether Trp or 5F-Trp (this study) was used for protein synthesis. Cofactor activation of VIIa by 5F-Trp labeled wild-type and mutant proteins and apparent binding affinities were measured by chromogenic assay based on production of Xa by the cofactor:VIIa complex as described previously.<sup>14</sup>

### Assessment of Analog Incorporation

Incorporation of F-Trp analogs is often <100% efficient and depends on the method of incorporation.<sup>4</sup> We used two approaches to assess the level of 5F-Trp incorporation into the wild-type and mutant sTF proteins. The first involved fitting absorbance spectra of the proteins, denatured at neutral pH in 6 M Gdm·Cl, by LINCS analysis.<sup>25</sup> This approach makes use of the absorption spectra of N-acetyl-Trp-amide, N-acetyl-Tyr-amide, and 5F-Trp as the basis set spectra for Trp, Tyr, and 5F-Trp residues. To recover the correct Tyr-to-Trp ratio from LINCS analysis of proteins or peptides of known composition containing Trp analogs, such as 5-hydroxytryptophan or 7-azatryptophan,

it was necessary to block the  $\alpha$ -amino groups of the analogs. However, 5F-Trp zwitterion in 6 M Gdm·Cl provided a satisfactory absorbance basis spectrum for 5F-Trp-containing sTF. The second approach to estimation of 5F-Trp incorporation was analysis of the protein mass spectrum by comparing relative peak heights of each appropriate molecular weight species, assuming each 5F-Trp residue will contribute an additional 18 amu to the protein molecular weight. Electrospray mass spectrometry (ESMS) and liquid chromatography ESMS (LC ESMS) were performed at the W. Alton Jones Cell Science Center, with use of a Perkin-Elmer Sciex API 300 triple quadrupole mass spectrometer (Concord, Thornhill, Ontario, Canada) fitted with an articulated ion spray plenum and an atmospheric pressure ionization source.

### Ultraviolet Absorption and Circular Dichroism Spectroscopies

Absorption spectra were measured at room temperature with a dual-beam Hitachi U-3210 spectrophotometer. The concentrations of wild-type and mutant proteins were determined by their molar extinction at 280 nm, as described,<sup>14</sup> after taking into account the fractional incorporation of 5F-Trp determined by the LINCS analysis and mass spectroscopy as described above. Circular dichroism spectra were obtained by using a JASCO J-500A spectrophotometer. A thermostated cell holder built in the laboratory provided temperature control.

### <sup>19</sup>F NMR Spectroscopy

<sup>19</sup>F NMR spectra were obtained at 470 MHz on a Varian Unity 500 at 25°C and 40°C using a triple-resonance probe with the center proton coil tuned to fluorine. A 12-MHz spectral width, 16 K data points, 60° pulse width, and a relaxation delay of 0.5 s were used for data collection. The processing parameters included either 25 or 10 Hz line broadening. The protein samples, in 0.1 M NaCl, 0.05 M Tris, pH 7.4 (TBS) buffer with 10% D<sub>2</sub>O (v/v) as the lock solvent, were between 2.5 and 20 mg/mL, and 3F-phenylalanine (3F-Phe) was used as an external standard (−38.0 ppm relative to trifluoroacetic acid). The mole fraction of D<sub>2</sub>O varied between 10 and 90% for the SIIS experiments. Stock solutions of 100 mM GdCl<sub>3</sub>/500 mM EDTA or 100 mM Gd:DTPA (adjusted to pH 7.1) were used for the line-broadening experiments, which were performed by sequential addition of the gadolinium complexes.

## RESULTS AND DISCUSSION

### Effects of 5F-Tryptophan Incorporation and Trp-to-Phe Mutations on sTF Function

As reported previously,<sup>14</sup> the mutant proteins did not express as well as wild-type sTF. Both LINCS analysis and mass spectra showed that replacement of Trp by 5F-Trp was less efficient in the four single Trp mutants than in wild-type sTF. This suggests that with less efficient protein expression, enough Trp is available after induction to compete efficiently with the analog during charging of Trp tRNA by tryptophanyl-tRNA synthetase. Nevertheless, the minimum degree of incorporation was ≈70%, and

incorporation in wild-type sTF was close to 100%. The apparent equilibrium dissociation constant ( $K_D$ ) at 25°C for binding of 5F-Trp containing sTF to VIIa was 2 nM ( $\Delta G = -11.8 \pm 0.3$  kcal mol $^{-1}$ ), the same as reported previously for unlabeled sTF.<sup>14</sup> The analog labeled mutants W14F and W158F had binding affinities for VIIa that were indistinguishable from that of wild-type sTF, whereas binding affinity of labeled W25Y was reduced about 4-fold, and the binding affinity of labeled W45F was reduced more than 20-fold. These affinities are essentially the same as reported previously for the unlabeled mutants,<sup>14</sup> showing that 5F-Trp incorporation has no observable effect on binding to VIIa. Activation of VIIa catalysis, as measured by conversion of factor X to Xa at saturating concentrations of sTF, was the same as observed previously<sup>14</sup>; the wild-type and mutant cofactor complexes exhibited essentially equivalent catalytic efficiencies with catalytic rate constants of 3–4 min $^{-1}$  ( $\pm 25\%$ ). Thus, the 5F-Trp labeled wild-type protein and mutants W14F, W45F, and W158F, which were used to provide the  $^{19}\text{F}$  NMR assignments, as well as W25Y, which was not expressed in quantities sufficient for  $^{19}\text{F}$  NMR, were indistinguishable from the unlabeled proteins in either VIIa binding or activation.

#### $^{19}\text{F}$ NMR Assignments for 5F-Tryptophan Residues in sTF

The spectrum of wild-type 5F-Trp-containing sTF was compared with the individual spectra of the functional 5F-Trp-containing single Trp replacement mutants. Assignment of Trp resonances was made by observing which peak in the wild-type spectrum was eliminated in the spectrum of each mutant (Fig. 2). Four Trp resonances can be identified in the wild-type sTF spectrum. There are two well-resolved peaks at  $-45.35$  and  $-47.16$  ppm, and a peak composed of two overlapping resonances with apparent maxima at  $-47.83$  and  $-47.92$  ppm. The ratio of the integrated peak areas corresponding to the two resolved and the two overlapping peaks is 1:1:2, indicating that each 5F-Trp residue contributes equally to the signal.

The spectra of three mutants (W14F, W45F, and W158F) were sufficient to assign all four 5F-Trp resonances (Table I). The ratio of the integrals of the peaks is 1:1:1 in the spectra of mutants W14F and W45F and 1:2 in the spectrum of W158F. Thus, it is evident that even though analog incorporation is less efficient in the mutants than in the wild-type protein (see Table II), replacement of Trp residues by 5F-Trp is random. The W14F and W45F spectra show that the overlapping resonances in the wild-type and W158F spectra must be due to 5F-Trp14 and 5F-Trp45. In addition, it is evident that the resonance at approximately  $-47.9$  ppm is due to 5F-Trp14. The loss of the central peak near  $-47.2$  ppm in the W158F spectrum indicates that this missing peak is due to 5F-Trp158. Thus, the remaining peak near  $-45.4$  ppm is due to 5F-Trp25. By increasing the sample temperature from 25°C to 40°C, the resonances become narrower because of the reduction in the rotational correlation time of the protein. The line narrowing improves the resolution of the two overlapping resonances, which have maxima at  $-47.61$  and  $-47.82$

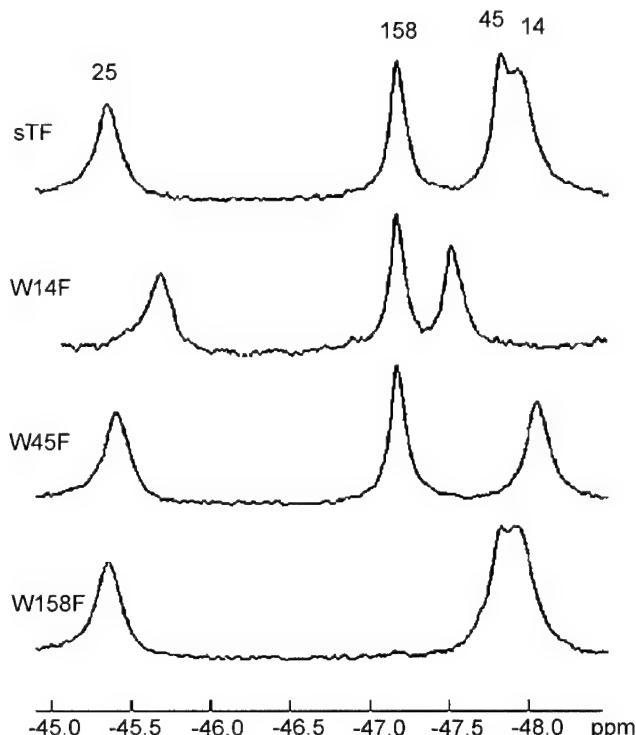


Fig. 2.  $^{19}\text{F}$  NMR spectra (470 MHz, proton-decoupled) of wild-type, W158F, W45F, and W14F sTF, obtained as described in Materials and Methods. The sample buffer was TBS (pH 7.4), 1 mM EDTA, and 1 mM  $\text{NaN}_3$ , with 10%  $\text{D}_2\text{O}$  as the solvent lock. 3F-Phenylalanine was used as an external standard ( $-38.0$  ppm relative to trifluoroacetic acid).

TABLE I.  $^{19}\text{F}$  Chemical Shifts (ppm) for 5F-Trp Residues in Wild-Type sTF and Trp-to-Phe Mutants

Residue	sTF	W14F	W45F	W158F
Trp14	$-47.92$		$-48.05$	$-47.92$
Trp25	$-45.35$	$-45.68$	$-45.41$	$-45.35$
Trp45	$-47.83$	$-47.51$		$-47.83$
Trp158	$-47.16$	$-47.16$	$-47.16$	

ppm, and there is no effect on the chemical shifts of the other peaks (see Fig. 3). To check whether increasing the temperature to 40°C perturbs the structure of sTF, the protein stability as a function of temperature was monitored separately by circular dichroism (CD). The CD spectrum remains constant up to about 50°C (data not shown), indicating that there are no significant changes in secondary structure in the temperature range used for the  $^{19}\text{F}$  NMR experiments. Above 50°C, the protein denatures.

#### Effects of Trp-to-Phe Mutations on $^{19}\text{F}$ NMR Spectrum of 5F-Trp Residues in sTF

As shown in Figure 1, domain I of sTF contains Trp14, Trp25, and Trp45, whereas domain II contains a single Trp, Trp158. The resonance corresponding with 5F-Trp158 is unaffected by mutation of either Trp14 or Trp45. Also, the resonances of the 5F-Trp residues at positions 14, 25, or 45 in the wild-type sTF spectrum are not affected by

TABLE II. Estimation of 5F-Trp Incorporation in Wild-Type sTF and Single Trp-Replacement Mutants by LINCS Analysis and by Mass Spectrometry<sup>†</sup>

Protein	LINCS (%)	Mass spectrum (%)
Wild-type	100	94/100
W14F	77	74
W25Y	72	68/73
W45F	83	73
W158F	79	74

<sup>†</sup>Percent incorporation of 5F-Trp in mutant and wild-type proteins was determined as described in Materials and Methods. Estimated precision of values obtained from LINCS is  $\pm 8\%$ . Values obtained from mass spectrometry represent results of individual measurements.

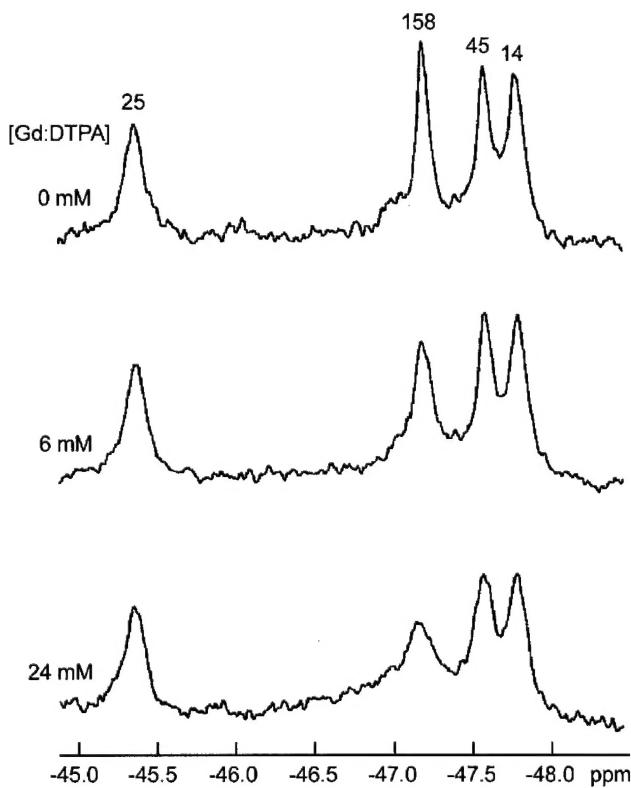


Fig. 3. Perturbation of <sup>19</sup>F NMR spectra of wild-type sTF by Gd:DTPA at 40°C. The sample buffer was TBS (pH 7.4), 1 mM EDTA, and 1 mM Na<sub>3</sub>N. Spectra shown are for 0, 6, and 24 mM Gd:DTPA.

mutation of Trp158. Thus, the Trp-to-Phe mutations in one domain have no discernable effect on the 5F-Trp resonances of the other domain. However, significant changes in chemical shifts were observed in the resonances of the 5F-Trp residues in domain I of the mutants W14F and W45F compared with the corresponding resonances in the spectrum of wild-type sTF (Fig. 2). The changes in the resonances associated with 5F-Trp25 ( $-0.3$  ppm) and 5F-Trp45 ( $+0.3$  ppm) in the spectrum of W14F are larger than those associated with 5F-Trp14 ( $-0.1$  ppm) and 5F-Trp25 ( $-0.1$  ppm) in the spectrum of W45F. These

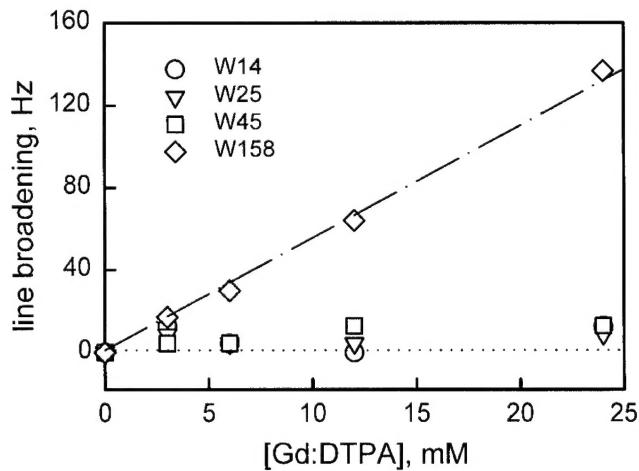


Fig. 4. Paramagnetic line broadening of 5F-Trp resonances in sTF as a function of Gd:DTPA concentration under the conditions described in Figure 3.

changes in the chemical shifts of the domain I mutants suggest that the Trp-to-Phe mutations in fact perturb the protein structure, even though these perturbations have no apparent effect on function.

In the case of Trp-to-Phe mutations, local structural rearrangements involving interactions with the aromatic ring might be expected for several reasons. These include the reduction in ring volume, reduction in ring dipole moment, and elimination of the possibility of N1 ring hydrogen bonding.<sup>26</sup> It is evident from the X-ray crystal structures of sTF<sup>15-17</sup> that the local environments of Trp14, Trp25, and Trp45 in domain I share important local elements of structure. For example, the Leu23 side chain is sandwiched between the aromatic rings of Trp14 and Trp25, which are the two most buried residues. Also, the Ala73 main chain atoms abut the aromatic ring of Trp45, whereas its methyl side chain abuts the aromatic ring of Trp14 and the methyl groups of the Leu23 side chain. Thus, shared structural elements contribute to the local environments of each of the Trp residues in domain I. As a result, mutation of any one of the three Trp residues is likely to perturb the local environments of the other two. The larger change in the 5F-Trp25 resonance of mutant W14F compared with that of mutant W45F is consistent with the shared components of the local environment of a buried 5F-Trp residue being perturbed, in particular, by the reduction in occupied volume of a nearby buried, mutated neighbor. However, the fact that the single Trp replacement mutants all form functional complexes with VIIa indicates that the structural perturbations are sufficiently subtle so that the essential features of the wild-type cofactor interactions with VIIa are maintained.

#### Solvent Accessibility and the Local Environments of the 5F-Trp Residues in sTF

The four resolved resonances in the <sup>19</sup>F NMR spectrum of wild-type sTF indicate that the local environment of each 5F-Trp residue has chemically unique characteris-

**TABLE III. Solvent Accessibilities of Trp Residues by X-ray Crystallography, Fluorescence, Absorption, and <sup>19</sup>F NMR Spectroscopies**

Residue	Water accessible area <sup>a</sup>	Fluorescence <sup>b</sup>	Absorbance <sup>c</sup>	<sup>19</sup> F NMR
Trp14	39 Å <sup>2</sup>	Blue-shifted spectrum, iodide $k_q = 9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	Narrow vibrational bands, red-shifted spectrum	Broad (?) resonance, inaccessible to perturbants
Trp25	16 Å <sup>2</sup>		Narrow vibrational bands, red-shifted spectrum	Broad resonance, inaccessible to perturbants
Trp45	86 Å <sup>2</sup>	Intermediate spectral shift, iodide $k_q = 4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$	Broad vibrational bands, red-shifted spectrum	Intermediate width resonance, inaccessible to perturbants
Trp158	41 Å <sup>2</sup>		Broad vibrational bands, red-shifted spectrum	Narrow resonance, intermediate perturbant accessibility

<sup>a</sup>The solvent accessible areas were calculated by using an algorithm developed by Kabsch and Sander,<sup>30</sup> which assesses the number of water molecules (using a radius of 1.40 Å) that can come in contact with a particular part of the protein, such as a Trp residue.

<sup>b</sup>Fluorescence shifts are defined with respect to emission spectra of Trp model compounds in water (red-shift or low energy) or a low-dielectric solvent such as dioxane (blue-shift or high energy). The bimolecular constant,  $k_q$ , values reported are from Hasselbacher et al.<sup>14</sup> The  $k_q$  for solute quenching of free Trp by iodide is about  $3.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ .

<sup>c</sup>Absorption shifts and vibrational band widths also are defined according to spectra in water (blue-shift or high energy, broad vibrational bands) or in a low-dielectric solvent (red-shift or low energy, narrow vibrational bands).

tics. Because the individual resonances of the 5F-Trp residues are resolved, it is possible to assess directly the relative solvent exposure of each residue either by observing the degree of solvent-induced isotope-shift (SIIS), using D<sub>2</sub>O, or the extent of paramagnetic line broadening. For a fully solvent-exposed 5F-Trp resonance, the SIIS is about 100 Hz.<sup>27</sup> SIIS experiments were performed at both 25°C and 40°C in a perturbant concentration series from 0 to 90% D<sub>2</sub>O. Over this range, the chemical shifts of all of the 5F-Trp residues except 5F-Trp158 remained the same within the estimated experimental error of  $\pm 15$  Hz. By comparison, the shift of 5F-Trp158 was calculated to be in the range 40–50 Hz for 100% D<sub>2</sub>O. This shift is about half that of the free amino acid 3F-Phe, indicating that the fluorine atom of 5F-Trp158 is significantly less solvent accessible than that of free 3F-Phe but more accessible than that of the other 5F-Trp residues.

The <sup>19</sup>F NMR line broadening due to interaction with paramagnetic metals occurs via spin-spin coupling between the nuclei, which has an inverse sixth power distance dependence, becoming effective within distances of a few angstroms.<sup>5</sup> Perturbation by chelated paramagnetic ions, therefore, provides a measure of distance from a fluorine atom. For example, Luck and Falke<sup>28</sup> used Gd:EDTA perturbation of a 5F-Trp residue in the sugar-binding site of a galactose-binding protein to investigate the open cleft of the ligand-binding site. The results from the <sup>19</sup>F NMR line-broadening experiments with the paramagnetic solutes Gd:DTPA (no net charge) and Gd:EDTA (one negative charge) corresponded closely with those from SIIS experiments in demonstrating the relative solvent exposure of 5F-Trp158, the only residue showing perturbation in either experiment. Titrations at 25°C of wild-type sTF and mutants W14F and W45F were performed with both Gd:EDTA and Gd:DTPA, and a titration at 40°C of wild-type sTF was performed with Gd:DTPA. As an example of the effect of the paramagnetic broadening, the latter titration is shown in Figure 3, and the results are summarized in Figure 4. All of the paramagnetic ion titration data show that the resolved resonance of 5F-Trp158 broadens

significantly, as measured by increased full width at half height, whereas the resonances of 5F-Trp25, 5F-Trp14, and 5F-Trp45 appear to be essentially unaffected. These results show that the fluorine atom of only 5F-Trp158 is accessible to either Gd:EDTA or Gd:DTPA. The observation that both paramagnetic ions yield equivalent results indicates that negatively charged residues do not interfere with the line broadening.

Hasselbacher et al.<sup>14</sup> compared the solvent accessibilities of the four Trp residues in sTF, calculated from the X-ray crystal structure,<sup>15</sup> with that assessed by fluorescence and difference absorption spectra. The results of this comparison are summarized in Table III along with the present results from <sup>19</sup>F NMR. On the basis of the X-ray crystal structure, Trp25 is the most buried residue, Trp14 and Trp158 have about equivalent solvent exposure, whereas Trp45 is the most exposed residue. Because Trp45 and Trp14 are the dominant fluorescence emitters, their relative solvent accessibilities could be assessed by the Stoke's shift of their emission spectra and by emission quenching using iodide. By these criteria and the characteristics of their difference absorption spectra, the indole ring of Trp45 is partially exposed to solvent, whereas that of Trp14 is essentially buried within the protein matrix, which also is indicated by their calculated solvent accessibilities. However, because Trp25 and Trp158 are essentially nonfluorescent, the conclusion that the indole ring of Trp158 is partially exposed to solvent, whereas that of Trp25 is buried was derived solely on the basis of difference absorption spectra. Although the overall shift of the absorption spectrum and resolution of the vibrational bands provide information about the local environment and solvent interactions of the indole ring,<sup>29</sup> the absorption properties are more difficult to resolve and much less sensitive than the fluorescence properties. Fluorescence emission has the advantage of larger energy shifts and intensity changes including the marked susceptibility to iodide quenching, which is a measure of solvent exposure.<sup>1</sup> Taken together, the difference fluorescence and absorption spectra suggest that the four Trp residues can be sepa-

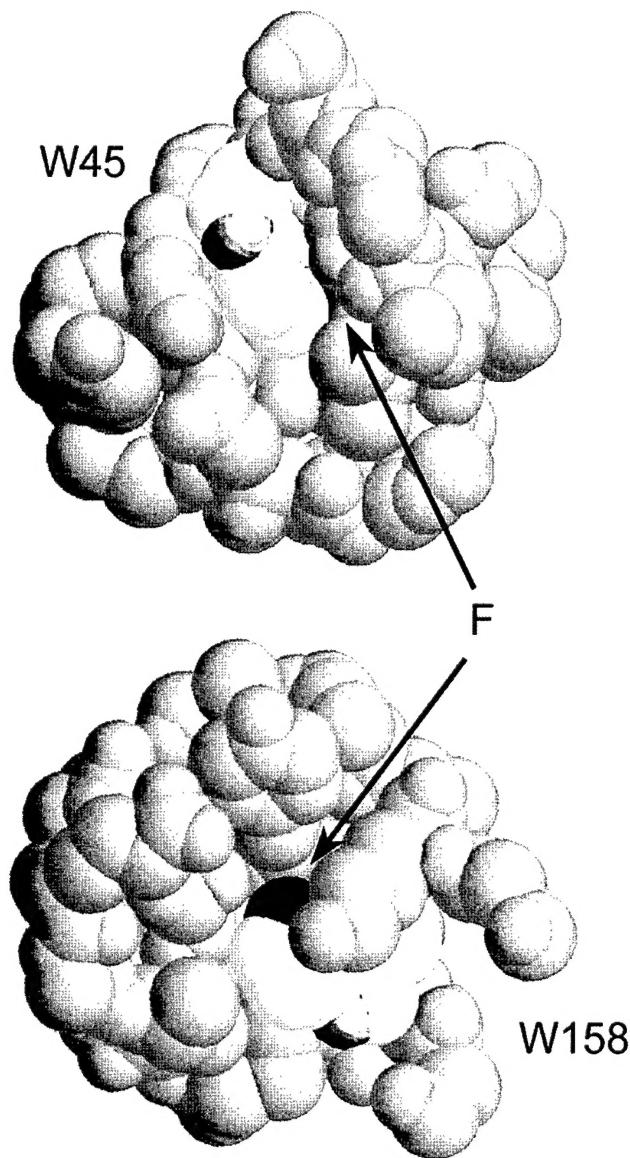


Fig. 5. Rasmol<sup>31</sup> space-filling representation of the local environments of Trp 45 and Trp 158 in sTF based on the 1.7 Å resolution X-ray crystal structure by Muller et al.<sup>16</sup> The protein atoms within 10 Å of the C-5 atom of each indole ring are shown in gray, but solvent molecules are not shown. The indole ring carbon atoms of each Trp residue are shown in white except for atom C-5, which is indicated by the arrow and black to indicate the position where the indole ring is fluorinated, and the indole nitrogen atom is speckled. It should be noted that although C-5 of Trp 45 is essentially buried in the protein matrix, C-5 of Trp 158 appears partially exposed to solvent. Both nitrogen atoms are exposed.

rated into two classes for solvent exposure. Accordingly, Trp14 and Trp25 are considered "buried" residues, essentially inaccessible to bulk solvent, whereas Trp45 and Trp158 are considered "partially buried" residues—partially buried in the protein matrix and partially exposed to bulk solvent.

As shown in Figure 5, the partial exposure of Trp45 and Trp158, indicated by the difference fluorescence and absorption results of Hasselbacher et al.<sup>14</sup> is borne out by the

X-ray crystal structure.<sup>15</sup> The <sup>19</sup>F NMR data essentially assess the local environment and solvent accessibility of the fluorine atom, and in the absence of any other information, the paramagnetic line-broadening and SIIS results would suggest that the indole ring of 5F-Trp45 is buried, whereas that of 5F-Trp158 is partially exposed. The <sup>19</sup>F NMR results, however, are at variance neither with the X-ray crystal structure nor the results of Hasselbacher et al.,<sup>14</sup> if the conformation of 5F-Trp and Trp are identical in sTF. As seen in Figure 5, the fluorine atom at the 5-position on the indole ring of 5F-Trp158 would be exposed to solvent, whereas that of 5F-Trp45 would be well shielded by the protein matrix. It also should be noted that the resonance of 5F-Trp158 is significantly more narrow (35–40%) than those of other 5F-Trp residues (40°C data), consistent with a greater exposure to solvent.<sup>2–4</sup>

The comparison of the fluorescence of the Trp residues in sTF with <sup>19</sup>F NMR of 5F-Trp residues in the same protein, including information about the local environments obtained from the X-ray crystal structure, highlights the advantages of combining information from fluorescence with <sup>19</sup>F NMR. Whereas fluorescence reports information about the local environment of essentially the entire indole ring, <sup>19</sup>F NMR reports information that is dominated by the solvent accessibility of a specific atom. The inherent sensitivity of the <sup>19</sup>F chemical shift to the local environment often is sufficient to resolve the peak resonances of individual Trp residues. By contrast, the overall fluorescence (and absorption) spectrum of Trp residues in different protein environments does not yield resolved individual spectra. However, as described,<sup>14</sup> difference spectral methods making use of single Trp replacement mutants can separate the individual contributions of both absorption and fluorescence. <sup>19</sup>F NMR also can take advantage of the possibility of using fluorine substitutions at different positions on the indole ring. Thus, as confirmed by the X-ray crystal structure of sTF, "solvent accessibility" revealed by combining results of <sup>19</sup>F NMR and fluorescence can help define for proteins in solution the spatial relationship of the indole ring of Trp residues with respect to the protein matrix and the bulk solvent.

A special feature of <sup>19</sup>F NMR is accessibility of this technique to individual molecules and molecular assemblies up to 100 kDa.<sup>4</sup> This is of special interest to us because sTF and VIIa together form a complex of about 75 kDa, and currently we are investigating effects on their interaction that result from occupation of the active site of VIIa. Although <sup>1</sup>H NMR in combination with multidimensional NMR techniques can provide greater resolution than that obtained by <sup>19</sup>F NMR, <sup>1</sup>H NMR generally is limited to molecules smaller than 30 kDa.<sup>4</sup> A subsequent article will report the fluorescence properties of sTF containing 5F-Trp. This analog has an absorption spectrum shifted to lower energy (red-shifted) relative to Trp absorption. This red-shift facilitates selective excitation of the analog fluorescence, which has the unique advantage that 5F-Trp-labeled sTF can be isolated spectroscopically from VIIa, and similarly, 5F-Trp peptide inhibitors can be used

as <sup>19</sup>F NMR and fluorescence probes for the active site of VIIa.

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### REFERENCES

- Eftink MR. The use of fluorescence methods to monitor unfolding transitions in proteins. *Biophys J* 1994;31:516-523.
- Gerig JT. Fluorine nuclear magnetic resonance of fluorinated ligands. *Methods Enzymol* 1989;177:3-23.
- Gerig JT. Fluorine NMR of proteins. *Prog NMR Spectrosc* 1994;26:293-370.
- Danielson MA, Falke JJ. Use of <sup>19</sup>F NMR to probe protein structure and conformational changes. In: Stroud RM, Hubbel WL, Olson WK, Sheetz MP, editors. *Annual review of biophysics and biomolecular structure*. Palo Alto: Annual Reviews, Inc.; 1996. p 163-195.
- Carrington A, McLachlan AD. *Introduction to magnetic resonance with applications to chemistry and chemical physics*. New York: Harper & Row; 1967.
- Brownlee RTC, Taft RW. A CNDO/2 Theoretical study of substituent effects on electronic distributions in fluorine molecular orbitals: comparison with meta- and para-substituent fluorine nuclear magnetic resonance shifts. *J Am Chem Soc* 1970;92:7007-7019.
- Bach R. Initiation of coagulation by tissue factor. *CRC Crit Rev Biochem* 1988;23:339-368.
- Nemerson Y. Tissue factor and hemostasis [published erratum appears in *Blood* 1988, 71:1178]. *Blood* 1988;71:1-8.
- Nemerson Y. Tissue factor: then and now. *Thromb Haemost* 1995;74:180-184.
- Edgington TS, Mackman N, Brand K, Ruf W. The structural biology of expression and function of tissue factor. *Thromb Haemost* 1991;66:67-79.
- Davie EW. Biochemical and molecular aspects of the coagulation cascade. *Thromb Haemost* 1995;74:1-6.
- Østerud B. Tissue factor: a complex biological role. *Thromb Haemost* 1997;78:755-758.
- Nemerson Y, Gentry R. An ordered addition, essential activation model of the tissue factor pathway of coagulation: evidence for a conformational cage [published erratum appears in *Biochemistry* 1987, 26(3):974]. *Biochemistry* 1986;25:4020-4033.
- Hasselbacher CA, Rusinova E, Waxman E, Rusinova R, Kohanski RA, Lam W, Guha A, Du J, Lin TC, Polikarpov I, Boys CWG, Nemerson Y, Konigsberg WH, Ross JBA. Environments of the four tryptophans in the extracellular domain of human tissue factor: comparison of results from absorption and fluorescence difference spectra of tryptophan replacement mutants with the crystal structure of the wild-type protein. *Biophys J* 1995;69:20-29.
- Harlos K, Martin DM, O'Brien DP, Jones EY, Stuart DI, Polikarpov I, Miller A, Tuddenham EG, Boys CWG. Crystal structure of the extracellular region of human tissue factor [published erratum appears in *Nature* 1994, 371: 720]. *Nature* 1994;370:662-666.
- Muller YA, Ultsch MH, de Vos AM. The crystal structure of the extracellular domain of human tissue factor refined to 1.7 Å resolution. *J Mol Biol* 1996;256:144-159.
- Muller YA, Ultsch MH, Kelley RF, de Vos AM. Structure of the extracellular domain of human tissue factor: location of the factor VIIa binding site. *Biochemistry* 1994;33:10864-10870.
- Broze GJ, Jr., Majerus PW. Purification and properties of human coagulation factor VII. *J Biol Chem* 1980;255:1242-1247.
- Miletich JP, Broze GJ, Jr., Majerus PW. Purification of human coagulation factors II, IX, and X using sulfated dextran beads. *Methods Enzymol* 1981;80:221-228.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning, a laboratory manual*. New York: Cold Spring Harbor Press; 1989.
- Ross JBA, Szabo AG, Hogue CWV. Enhancement of protein spectra with tryptophan analogs: fluorescence spectroscopy of protein-protein and protein-nucleic acid interactions. *Methods Enzymol* 1997;278:151-190.
- Snavely MD, Florer JB, Miller CG, Maguire ME. Magnesium transport in *Salmonella typhimurium*: expression of cloned genes for three distinct Mg<sup>2+</sup> transport systems. *J Bacteriol* 1989;171:4752-4760.
- Waxman E, Ross JBA, Laue TM, Guha A, Thiruvikraman SV, Lin TC, Konigsberg WH, Nemerson Y. Tissue factor and its extracellular soluble domain: the relationship between intermolecular association with factor VIIa and enzymatic activity of the complex. *Biochemistry* 1992;31:3998-4003.
- Hasselbacher CA, Rusinova R, Rusinova E, Ross JBA. Spectral enhancement of recombinant proteins with tryptophan analogs: the soluble domain of human tissue factor. In: Crabb JW, editor. *Techniques in Protein Chemistry*. New York: Academic Press; 1995; p 349-356.
- Waxman E, Rusinova E, Hasselbacher CA, Schwartz GP, Laws WR, Ross JBA. Determination of the tryptophan:tyrosine ratio in proteins. *Anal Biochem* 1993;210:425-428.
- Huang YT, Rusinova E, Ross JBA, Senechal DF. An aromatic stacking interaction between subunits helps mediate DNA sequence specificity: operator site discrimination by phage λ cI repressor. *J Mol Biol* 1997;267: 403-417.
- Luck LA, Falke JJ. <sup>19</sup>F NMR studies of the D-galactose chemosensory receptor. 2. Ca(II) binding yields a local structural change. *Biochemistry* 1991; 30:4257-4261.
- Luck LA, Falke JJ. Open conformation of a substrate-binding cleft: <sup>19</sup>F NMR studies of cleft angle in the D-galactose chemosensory receptor. *Biochemistry* 1991; 30:6484-6490.
- Strickland EH, Horwitz J, Kay E, Shannon LM, Wilcheck M, Billups C. Near-ultraviolet absorption bands of tryptophan: studies using horseradish peroxidase isoenzymes, bovine and horse heart cytochrome C, and N-stearyl-L-tryptophan n-hexyl ester. *Biochemistry* 1971;10:2631-2638.
- Kabsch W, Sander C. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 1983;22:2577-2637.
- Sayle RA, Milner-White EJ. RasMol: biomolecular graphics for all. *Trends Biochem Sci* 1995; 20:374-376.